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Contributions of Monoclonal Antibodies With Anti-Neural Cell Adhesion Molecule Like Activity to Peripheral Nerve Regeneration.

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CONTRIBUTIONS OF MONOCLONAL ANTIBODIES WITH ANTI-NEURAL CELL ADHESION MOLECULE LIKE ACTIVITY TO PERIPHERAL NERVE REGENERATION

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ABSTRACT

Tubes containing monoclonal antibodies with anti-neural cell adhesion molecule (N-CAM)-like activity were applied to transected sciatic nerves to attempt to perturb the recovery of muscle function. Physiological recordings were used to estimate the return of function. The decline of implanted antibody over 28 days was estimated. No significant immune responses were detected in response to the implanted material.

Electron microscopic and immunohistological analyses evaluated particular cellular disruptions in nerves due to the presence of these antibodies with anti-N-CAM like activity. Histological sections of fixed experimental nerves consistently revealed abnormal gaps between Schwann cells of regenerating nerves. This specific Schwann cell abnormality was not present in nerves of control animals and was no longer observed in experimental nerves after 60 days of survival. This time course was associated with antibody clearance and restoration of muscle function. We proposed that perturbed Schwann cell adhesive interactions disrupted the advance of neurites across nerve gaps and resulted in delayed regeneration. The data implicated N-CAM as a potential contributor to nerve regeneration.

INTRODUCTION

To regenerate an injured axon, a neuron must undergo a series of physiological rearrangements. It must reorganize its terminal membrane and cytoplasm to generate a motile structure called the growth cone, that will move in a directed manner to appropriate target cells and eventually form a synaptic terminal. The neuron must also produce the membrane, cytoplasm and cytoskeleton required by the elongating and expanding sprout.

In the peripheral nervous system of adult mammals, damaged axons can regenerate for many centimeters from the site of injury (Ramon y Cajal, 1928a,b). These regenerating axons are typically found within conduits of basement membrane and are often in contact with Schwann cells (Hillarp and Olivecrona, 1946). The basement membrane in the peripheral nervous system, like many other basement membranes, is comprised of laminin, fibronectin and other extracellular matrix materials. Purified fibronectin, collagen and laminin have been shown to stimulate neural outgrowth and to guide nerve processes under *in vitro* conditions (Woolley *et al.*, 1990). This growth requires binding of cell-surface receptors to extracellular matrix adhesive proteins and requires binding of regenerating axonal sprouts to Schwann cells (Hillarp and Olivecrona, 1946). In the regenerating neurite, there are multiple adhesive systems for both cell-cell and cell-matrix receptors that are present on neurons, Schwann cells and fibroblasts (Dellon, 1990).

The neural cell adhesion molecule (N-CAM) is a membrane glycoprotein that serves as a homophilic ligand in the formation of adhesions between cells (Hoffman and Edelman, 1983). It is expressed by several types of cells during embryogenesis including nerve, muscle and glial cells of the developing nervous system (Edelman, 1984). N-CAM-mediated adhesion is involved in many developmental events including axon guidance, segregation of cells into discreet layers, and the formation and innervation of muscles. Molecular interactions at the axon-Schwann cell interface that initiate the formation of myelin sheaths involve N-CAM and L1 adhesion molecule. Both N-CAM and L1 are present on the membrane of axons and Schwann cells before the onset of myelination, but are reduced and weakly detectable after myelination commences (Seilheimer and Schachner, 1988).

N-CAM is on Schwann cells and their basal laminae and may mediate Schwann cell adhesion (Daniloff *et al.*, 1986b). These cells adhere to one another during regeneration to form cords that guide regenerating nerves through an injured area. These proliferating Schwann cells align longitudinally within the confines of the basal lamina or endoneurial tube, creating a continuous column of cells called the bands of Büngner (Sunderland and Bradley, 1952; Thomas, 1963; Komiyama *et al.*, 1991). Schwann cells also adhere to the basal lamina as they grow toward the site of injury (Nathaniel and Pease, 1963; Ide *et al.*, 1983). N-CAM can be detected in the basement membrane of Schwann cells and

collagen fibrils of the endoneurium (Daniloff *et al.*, 1986a). It has been found that axon-Schwann cell interactions are characterized by the sequential appearance of cell adhesion molecules (CAMs) and myelin basic protein coordinated in time and space. It was deduced that N-CAM was involved in fasciculation, initial axon-Schwann cell interactions and the onset of myelination (Daniloff *et al.*, 1986b). Two to six days following transection, small diameter regenerating axons were found to be positive for N-CAM in regions where they made contact with one another or with Schwann cells. Large diameter axons showed negligible amounts of N-CAM. Fourteen days after transection, when regrowing axons were seen in the distal part of the transected nerve, N-CAM was observed where regrowing axons made contact with Schwann cells. Most Schwann cells that were associated with degenerating myelin also expressed N-CAM. During myelination, N-CAM expression is reduced and disappears in compacted myelin (Mirsky *et al.*, 1986; Martini and Schachner, 1988). N-CAM is also involved in the initial stages of nerve-muscle contact (Rieger *et al.*, 1985) but is not essential for the formation of electrophysiologically active synapses.

Overview of Nerve Structure

Peripheral nerve trunks are composed of bundles of neurons, axons and connective tissue elements. Most nerves contain motor and sensory fibers; the latter conduct electrical impulses at a faster rate than do motor fibers. The presence of myelin profoundly enhances the velocity of impulse transmission.

Each myelinated fiber has a compact myelin sheath, which is composed of a lipid and protein bilayer. This sheath is formed by a Schwann cell wrapping spirally around the axon. Individual Schwann cells meet at the nodes of Ranvier, where small gaps in the myelin exist. At these nodes of Ranvier, the axon is surrounded only by the Schwann cell basal lamina. Small bundles of nonmyelinated axons can be encircled by a single Schwann cell, but no myelin is present (Kuczynski, 1980).

There are three layers of connective tissue in a nerve: endoneurium, perineurium, and epineurium. All individual axons are covered by endoneurium. This covering gives tensile strength to the nerve and promotes resistance to internal axonal pressure. Bundles of nerve fibers form fasciculi and are encircled by perineurium. The perineurium can be sutured in order to anastomose severed nerves (Kline and Kahn, 1982). Thick, outer connective tissue, called epineurium, covers the nerve trunk; epineurium has extensions that separate fasciculi and blend with the perineurium (Kucynski, 1980). This epineurium can be used to manipulate the nerve during repair and is a frequent site of suture placement in anastomosis of transected nerves (Sunderland, 1980; Braun, 1982).

Injuries to Nerves

Mammalian peripheral nerve fibers are capable of repair by regeneration after injury. Conditions for successful regeneration will be best after nerve crush; the Schwann cell basement membrane tubes (endoneurial tubes) remain

intact and this gives the injured axons a measure of protection from extracellular fluid and products of tissue damage at the injury site. The tubes also serve to contain the Schwann cells needed to support the regenerating axon sprouts and to guide them into the distal stump and then on to reinnervate peripheral targets (Horch, 1979; Horch and Lisney, 1981).

Recovery is more likely to occur if axons are simply crushed (Nicholson and Seddon, 1957) or have a very short (less than 5 mm) interstump gap to cross (McQuarrie, 1986). This regeneration distal to the cell body is more likely to fail if the interstump gap is greater than 1 cm and associated with soft tissue damage. Even though reactive axonal sprouting is an intrinsic neuronal response to injury, the subsequent reorganization of these axonal sprouts does not occur unless Schwann cells are present (Aguayo and Bray, 1980; Lisney, 1989). In normal, young adult rats sustaining experimental nerve gaps of 10mm, there are significant attempts to regenerate and some recovery occurs (Gibson and Daniloff, 1989b; Madison *et al.*, 1987).

Transection of peripheral nerves causes a breakdown of myelin in the distal stump. This results in macrophage recruitment to remove myelin debris from this area. It has been suggested that these macrophages interact with Schwann cells and may be a source of Schwann cell mitogens (Scheidt and Friede, 1987).

During the injury response, Schwann cells proliferate. If the distal stump is separated from the proximal stump, Schwann cells co-migrate with regrowing axons. Schwann cells also respond to axonal cues by transient upregulation or re-expression of molecules which provide a favorable environment for axonal extension. They also attract bundles of regrowing axons and their associated Schwann cells across interstump gaps up to 1 cm in length (Ramon y Cajal, 1928b).

Schwann cell basal lamina is similar to basal laminae elsewhere, in that it contains molecules such as laminin and fibronectin, which are potent promoters of neurite growth in culture (Rogers *et al.*, 1983; Bozyczko and Horwitz, 1986). The behavior of Schwann cells inside the basal lamina depends on the presence of an axon. The initial breakdown products of axons after axotomy stimulate Schwann cell multiplication in preparation for phagocytosis of debris (Aguayo *et al.*, 1976). Subsequently, a regenerating axon is required for differentiation of the Schwann cell and production of myelin for the remyelination of the axon by the Schwann cell. The degree of remyelination is determined by the type of axon regenerating into the basal lamina (Hillarp and Olivecrona, 1946; Weinberg and Spencer, 1975).

The environment through which axons regenerate in the peripheral nervous system consists of Schwann cells and their basal laminae, fibroblasts, and collagen (Seckel, 1990). Early in regeneration, axonal debris, degenerating

myelin, and phagocytic cells are present (Fawcett and Keynes, 1990; Komiyama *et al.*, 1991). Other factors involved in regeneration include laminin, fibronectin, collagen, growth and trophic factors, cell-substrate adhesion molecules and cell-cell adhesion molecules (Fawcett and Keynes, 1990). External factors which influence regeneration include effects of a conditioning lesion and the affects of applied pulsed-electromagnetic fields (Aebischer, *et al.*, 1987). In principle, functional restorations following peripheral nerve regeneration and target reinnervation could be accomplished by either random outgrowth and secondary central adjustments of synaptic connections, or directed outgrowth towards the 'original' target areas as well as appropriate target structure reinnervation (McQuarrie, 1986). There are indications that specific factors are present in the distal stump of transected nerves. These factors are thought to attract axons of the corresponding proximal stump into the distal nerve stump (Ramon y Cajal, 1928a,b).

Nerve Degeneration

Nerve degeneration is a prerequisite for normal motor and sensory axon regeneration following an injury to a nerve (Bisby and Chen, 1990; Ramon y Cajal, 1928b). It leads to the removal and recycling of axonal and myelin-derived material and prepares the environment through which regenerating axons regrow. It is influenced by the peripheral connection of the injured nerve and by the distance the injured section is from the cell body (Gibson *et al.*, 1989a;

Delgado-Lezama and Muñoz-Martinez, 1990). Following axotomy, most surviving cell bodies undergo a variety of anatomical changes and modifications in gene expression and cellular metabolism.

The most obvious main morphological event is chromatolysis. Chromatolysis reflects a change in metabolic priority from that geared for the production of neurotransmitters needed for continuous synaptic activity, to the production of materials for axonal repair and growth. The cell must synthesize new messenger RNA, lipids and cytoskeletal proteins (Grafstein and McQuarrie, 1978). This process is characterized by a swelling of the cell soma, formation of a pyknotic nucleus and the dispersal of Nissl substance (Ramon y Cajal, 1928a,b). The latter is due to the disintegration of large granular condensations of rough endoplasmic reticulum (Grafstein and McQuarrie, 1978).

Distal to the site of injury, the severed axon and myelin sheath undergo Wallerian degeneration (Dyck *et al.*, 1984). The axon, without the continuity of supporting structures and trophic substances from the cell soma, begins to degenerate within 12 hours (Stoll *et al.*, 1989). The axon degenerates before the Schwann cell sheath and becomes irregularly swollen and beaded. The myelin sheath draws away from the axon and breaks apart. Both the axon and the myelin degenerate. Trophic factors accumulate and stimulate Schwann cells to replicate and move along the endoneurium (Ramon y Cajal, 1928a). Recently one of these trophic factors is reported to have been isolated (Ratner *et al.*,

1988). When they reach the site of the initial injury, the Schwann cells fuse with one another to form columns or bands, classically referred to as the bands of Büngner (Ramon y Cajal, 1928b). Some Schwann cells also become phagocytic in response to injury; ingesting fragments of the axon as well as myelin (Bunge, 1980a).

This degeneration continues distally toward the synapse where there is a progressive loss of synaptic vesicles; the nerve terminal is phagocytized away from the post-synaptic membrane by Schwann cells and phagocytes. A similar process occurs in unmyelinated nerve fibers (Lisney, 1989).

Nerve Repair

The two most common methods of surgical repair for injured motor nerves include direct suturing of nerve stumps for minor injuries and the transplantation of sensory nerve allografts when large gaps appear. Epineurium can be used to manipulate the nerve during repair and is the most frequent site of suture placement for repair of simple nerve transections when no gaps exist between nerve stumps (Sunderland, 1980; Braun, 1982). Allografts of sensory nerve autografts are often used to bridge gaps in severely injured nerves (Gibson *et al.*, 1989b). Overall results include variable recovery of function and the irrevocable loss of sensation associated with the removal of a sensory nerve (Kline and Kahn, 1982). The above procedures are attempted to maximize the number of axons that regenerate through the site of injury and grow back to the correct targets

(Millesi, 1990). Failure of axons to regenerate leads to poor sensory recovery and the innervation of incorrect targets leads to poor motor control as well as poor sensory recovery. It has been shown that implants of pure Schwann cells induce greater numbers of regenerating axons across nerve gaps than do autografts (Daniloff *et al.*, 1991).

Excessive tension on the nerve repair is believed to result in decreased regeneration and reduced overall function. Tension stretches the endoneurial tubes and results in physical disruption of nerve fibers, impairment of electrical conduction and compromise of the blood supply to the proximal and distal stumps (Miyamoto, 1979).

Sutureless methods of nerve transection repair have been investigated. These include the use of adhesives to join nerve ends; plasma clots to appose nerve stumps (Becker *et al.*, 1985), fibrin glue (Kuderna *et al.*, 1979) and carbon dioxide lasers to weld nerve stumps together (Tupper, 1980).

Entubulization, the implantation of nerve cuffs or guide tubes, has been shown to be an alternative to direct suture techniques (Molander *et al.*, 1983). The use of these nerve guide conduits is appropriate for injuries with significant nerve gaps (daSilva *et al.*, 1985). It is also pertinent for situations where direct realignment of nerve fascicles is impossible. Collagen-based nerve guide conduits are capable of supporting and maintaining axonal outgrowth, extension and maturation *in vivo* (Archibald, *et al.*, 1991).

Regeneration

Contributions of Schwann Cells

The proliferation of Schwann cells during Wallerian degeneration and subsequent regeneration has been well documented (Abercrombie and Johnson, 1946; Bradley and Ashbury, 1970; Pellegrino and Spencer, 1985; Ramon y Cajal, 1928b). The first wave of Schwann cell proliferation during Wallerian degeneration occurs 3-4 days after transection followed by an axonal regrowth stage occurring at 2-3 weeks (Pelligrino *et al.*, 1986). The role of Schwann cells and hematogenous macrophages in myelin degradation during Wallerian degeneration of the rodent sciatic nerve has been examined. It was found that before the appearance of adherent macrophages, the myelin sheath fragments into ovoids, small whorls of myelin debris appear within Schwann cell cytoplasm and the Schwann cell displays numerous lipid droplets (Stoll *et al.*, 1989). Myelin basic protein processed by macrophages was suggested to promote the proliferation of Schwann cells during Wallerian degeneration (Baichwal and DeVries, 1989). One Schwann cell mitogen has been isolated from regenerating nerves; this factor is an axonal surface proteoglycan-growth factor complex (Ratner *et al.*, 1988; Ratner, 1990). The adhesion and proliferation of Schwann cells is rapidly and transiently induced during Wallerian degeneration and this capacity is maintained longer in the presence of regenerating axons (Komiyama *et al.*, 1991).

This loss of axonal contact occurring with Wallerian degeneration is associated with an increased proliferation of Schwann cells (Clemence *et al.*, 1989). The proliferation of Schwann cells can also be stimulated by contact with newly regenerating axons or even with fractions of axonal membranes after Wallerian degeneration is complete (Salzer *et al.*, 1980; Wood and Bunge, 1975). If regeneration is prevented, Schwann cells in the distal stump initially bind to one another to form Schwann cell bands.

Contributions of Conditioning Lesions

An experimental conditioning lesion is a mild injury, usually involving a nerve compression, that is applied to a nerve prior to a second lesion. Many studies have shown that conditioning lesions increase the rate of regeneration (McQuarrie, 1986; Bisby and Pollock, 1983; Bisby, 1985; Oblinger and Lasek, 1984). This regeneration is associated with expression of genes and proteins from the cell body. In general, the proteins produced during regeneration are the same as those associated with axonal growth in embryos. These substances include tubulin, actin and growth associated proteins.

There are two main hypotheses regarding peripheral nerve regeneration after a conditioning lesion (Sjöberg and Kanje, 1990). The first is that the local environment surrounding the growth cone controls the rate of axonal elongation. The conditioning lesion causes Wallerian degeneration in the distal nerve, so that when the test lesion is made the axons are supposed to grow more rapidly along

pathways vacated by the previously degenerated axons. It is thought that changes in non-neuronal cells, Schwann cells in particular, and trophic factor production are responsible for neurite outgrowth (Bray *et al.*, 1978; Salzer *et al.*, 1980). The outgrowth of neurites starts within 3 hours after a crush injury (Sjöberg and Kanje, 1990). Therefore, this initial early outgrowth of axons; i.e., growth cone formation occurs without support from the cell body. This suggestion is supported by the observation that the distal segment of severed axons forms growth cones *in vitro* (Bray *et al.*, 1978; Shaw and Bray, 1977; Wessells *et al.*, 1978).

The second hypothesis is that outgrowth of axonal sprouts is enhanced in conditioned nerves because they are already activated when the second injury occurs. It is assumed that the nerve cell bodies have made necessary adjustments and axons have already synthesized materials necessary for regeneration (McQuarrie, 1986; Ducker *et al.*, 1969). It is not clear what signals between cell body and axon tip regulate the transition to regenerative growth, nor whether events at the axon tip are controlled in the same way as those at the cell body. The duration of the effect of the conditioning lesion is also not clear. An accepted model for the induction of the regenerative response is that the cell body invokes changes in a trophic factor normally derived from the target and is retrogradely transported to the cell body. However, the earliest regenerative sprouting at the axon tip can occur within a few hours of axotomy.

Theoretically, this is too rapid for the cell body to have been informed (Sjöberg and Kanje, 1990).

Investigators have determined at least two physical mechanisms which alter regeneration rate: cell body changes and the environment of the injured axons. Each may act in an additive fashion to produce a maximal conditioning lesion response (Bisby and Pollock, 1983). The nature of the control exerted by the axonal environment might be either the provision of providing vacant channels for axonal growth in the previously degenerated axons, or perhaps provision of a channel for a diffusible product of degeneration which stimulates the growth of axons (Bisby and Pollock, 1983).

Superimposition of the conditioning and test lesions on the sciatic nerve cause a positive reparative effect on motor neurons. When axons grew into predegenerated nerves their elongation rate was further increased above that obtained when the conditioning lesion was distal to the test lesion (Bisby, 1985). Recovery was accelerated by conditioning, with the entire recovery curve shifted to earlier time intervals in conditioned nerves, and the initial rate of recovery was greater. This observation was consistent with a more rapid elongation of axons along the peripheral nerve trunks and a more rapid invasion of the denervated muscles. This increased regeneration rate was associated with an earlier recovery of function (Bisby, 1985).

Axons also regenerate *in vitro* much more vigorously from explants taken during the period of embryonic axonal growth, or from older explants whose axons have previously been induced to regenerate (Collins and Lee, 1982). The enhanced regenerative response following a conditioning lesion may be due to the early availability in the axon of molecules associated with regeneration.

Contributions of Electromagnetic Fields

The influence of electric and electromagnetic fields on growth and differentiation of nerve tissue *in vitro* has been reported (Orgel *et al.*, 1984; Cooper and Schliwa, 1985). The effects of pulsed electromagnetic fields (PEMF) on nerve regeneration in neurite outgrowth and in animal nerve injury models have been the subject of many investigations (Orgel *et al.*, 1984; Aebischer *et al.*, 1987). In *in vitro* experiments, cultures treated with PEMF showed significant increases in neurite outgrowth relative to controls (Sisken *et al.*, 1990). *In vivo* exposure to PEMF before lesion production in rat sciatic nerve produced an increase in axonal sprouting after lesion production (Sisken *et al.*, 1989a,b). In studies on a nerve crush model, rats placed immediately after injury in a pulsed magnetic field showed a 22% increase in the rate of axonal sprouting relative to controls (Wilson and Jagadesch, 1976). This increase was found to be approximately equal to that obtained with collagen or growth hormone and was not dependent upon primarily invasive delivery systems. A comparable enhancement of regeneration was obtained when the animals were exposed daily

for seven days before lesioning. This response resembles the response described for a "conditioning" lesion (McQuarrie, 1986; Bisby and Pollock, 1983). The same effect was found in one transection model of rat sciatic nerve in which gait analysis was performed 4-6 months following transection. In that study PEMF signals were found to significantly improve performance relative to untreated controls.

Electromagnetic fields were also found to influence the synthesis of new polypeptides in a rat sciatic nerve transection model. PEMF altered the distribution pattern of polypeptides in the injured nerve. Transection injury alone changed the pattern indicating a "stress" response. Transection combined with PEMF reduced the stress response changes, altering the quantitative distribution of new polypeptides in the sciatic nerve (Welch *et al.*, 1983).

Contributions of Cell-Substrate Adhesion Molecules

Laminin and Fibronectin

The adhesion of growing neurite processes to collagen or laminin (Bunge *et al.*, 1980b) is an important determinant of neurite elongation, because growing fibers orient toward substrates that permit the greatest adhesiveness, and preferentially move upon them. Investigators have shown that both fibronectin and laminin increase the survival of sensory neurons *in vitro*, and when used in combination significantly enhanced neurite outgrowth (Baron-Van Evercooren *et al.*, 1982).

Laminin is a high molecular weight glycoprotein of MW 850,000 or greater and is composed of disulfide-bonded subunit chains of 400 and 200 kilodaltons. *In vivo*, laminin has been found in basement membrane throughout the body, including the glomerular basement membrane, vascular endothelial basement membrane and the layer underlying the epithelium of the skin (Foidart *et al.*, 1980). It promotes the attachment of epithelial cells to Type IV collagen (Terranova *et al.*, 1980) and can bind to glycosaminoglycans (Del Rosso *et al.*, 1981). Laminin promotes axon extension by interacting with axonal glycoproteins that are members of the integrin family of receptors. Antibodies against integrins inhibit the extension of central and peripheral axons on laminin or extracellular matrix substrates (Tomaselli *et al.*, 1988).

Fibronectin is a high molecular weight glycoprotein of 440 kilodaltons with two apparently similar disulfide-bonded chains of approximately 220 kilodaltons. It is found in plasma and is a major component of basal lamina, connective tissue and the extracellular matrix produced by fibroblasts. It is involved in adhesion of cells including fibroblasts and platelets to collagen *in vitro* (Ruoslahti *et al.*, 1981). In development, fibronectin may play a role in muscle morphogenesis and in the spatial organization of cells in the developing chick wing (Ruoslahti *et al.*, 1981). Surfaces coated with laminin or fibronectin stimulate a more rapid extension of neuronal processes than do serum or collagen-coated plastic (Pierce *et al.*, 1988).

Results from an *in vitro* study of Schwann cell adhesion and proliferation showed that Schwann cells from injured nerves possess binding sites for laminin and fibronectin, which may be partly responsible for the enhanced adhesion of Schwann cells *in vitro* (Komiyama *et al.*, 1991). Another *in vitro* study showed that neurons induced the laminin mRNA levels of Schwann cells by releasing a diffusible signal into culture medium (Bunge *et al.*, 1989). It is thought that regenerating axons induce the expression of laminin and possibly fibronectin receptors on Schwann cells (Carey *et al.*, 1983). These receptors are responsible for the enhanced adhesiveness of Schwann cells *in vitro*. Laminin and fibronectin appear to be similar in their ability to promote the adhesion of Schwann cells from injured nerves at different times after crush or transection injury.

The presence of laminin with little fibronectin in the endoneurium of mouse sciatic nerve indicates that laminin occurs *in vivo* in a position where it may play a role in the regeneration and myelinization of injured axons (Foidart *et al.*, 1980). Laminin is also present along pathways of axonal growth during development (Rogers *et al.*, 1983) and regeneration (Hopkins *et al.*, 1985).

The presence of fibronectin in the perineurium (Foidart *et al.*, 1980) indicates that fibronectin may play a role in the Schwann cell-neuron connective interactions (Chiu *et al.*, 1991; Martin and Timpl, 1987). It is also found at nodes of Ranvier (Terranova *et al.*, 1980).

A previous study reported that 1) both fibronectin and laminin are components of the mature peripheral nerve and are localized in distinct patterns; 2) antibodies to each protein differentially recognize Schwann cells (laminin) and fibroblasts (fibronectin) in tissue culture preparations; and 3) laminin is expressed by the Schwann cell prior to the development of a morphologically recognizable basal lamina (Cornbrooks *et al.*, 1983). They also provided evidence that Schwann cells in culture were capable of synthesizing laminin but not fibronectin. Cultures of Schwann cell tumors synthesize extracellular matrix components including fibronectin and laminin (Palm and Furcht, 1983), and it has been found that added laminin enhances Schwann cell attachment and cell growth and causes the cells to elongate (McGarvey *et al.*, 1984). The Schwann cell basal lamina is similar to other basal laminae, in that it contains molecules such as laminin and fibronectin, which are potent promoters of neurite outgrowth in culture (Bozyczko and Horwitz, 1986; Rogers *et al.*, 1983). Another study showed that an *in vivo* combination of fibronectin and laminin significantly enhances the regeneration of myelinated axons across a long nerve gap in the rat sciatic nerve (Woolley *et al.*, 1990).

N-Cadherin

Cadherins are transmembrane proteins and their cytoplasmic domain is highly conserved among different members of this molecular family. They are a molecular family that is essential for the calcium-dependent process of cell-cell

adhesion (Takeichi, 1988). Their mature form consists of 723 to 748 amino acids and has a single transmembrane domain that divides the molecules into the amino-terminal extracellular and the carboxy-terminal cytoplasmic domain (Shimoyama *et al.*, 1989). It was observed that cells expressing one type of cadherin, when mixed with cells expressing another type of cadherin and cultured in suspension, tended to aggregate separately (Takeichi *et al.*, 1985). It is a reasonable conclusion that cadherins interact with cadherins only in a homophilic manner (Takeichi, 1990). Cadherin-mediated adhesion is temperature dependent (Takeichi, 1990), whereas Ig superfamily-mediated adhesion is not (Hoffman and Edelman, 1983).

N-cadherin mediates the attachment of neurites to cells on the substratum. In cell cultures of N-cadherin-transfected cells, a vigorous extension of optic nerves took place. The growth cones of the axons attached only to the surface of transfected cells, and not to a culture dish (Tomaselli *et al.*, 1988).

N-cadherin has a major role in the initial contacts of nerve growth cones with Schwann cells (Takeichi, 1988). Sensory neuronal growth cones on a laminin substratum were inhibited from migrating onto the upper surfaces of Schwann cells by soluble antibodies to N-cadherin. This behavior was blocked reversibly when N-cadherin was inactivated by lowering the calcium concentration of the culture medium from 1 to 0.1 mM (Volk and Geiger, 1986).

Retinal growth cones were also blocked from crawling onto Schwann cells by lowered calcium levels. Electron microscope immunocytochemistry indicated that N-cadherin is abundant on growth cones, on Schwann cells and at points of growth cone-Schwann cell contact (Letourneau *et al.*, 1990). The above results indicate that N-cadherin has a significant role in growth cone migration onto Schwann cells, since lowered calcium and anti-N-cadherin antibodies reduced growth cone migration onto Schwann cells. It functions during the initial migration of growth cones onto the surfaces of Schwann cells.

Contributions of Cell-Cell Adhesion Molecules

Cell adhesion is one of five primary processes of development that include cell division, migration, differentiation, adhesion and death (Edelman, 1982a). Cell adhesion is also a critical component of nerve regeneration (Bunge, 1980a; Cornbrooks *et al.*, 1983).

Neural Cell Adhesion Molecule (N-CAM)

N-CAM is a high molecular weight cell surface glycoprotein that has been characterized extensively (Brackenbury *et al.*, 1977; Edelman and Chuong, 1982b; Edelman, 1984; Hoffman *et al.*, 1982; Rutishauser 1983,1984; Rutishauser and Goridis, 1986; Edelman, 1983). The basic protein backbone of rat N-CAM includes three major moieties that migrate at molecular weights of 115, 135, and 190 kilodaltons on sodium dodecyl sulfate - polyacrylamide (SDS-PAGE) gels (Edelman and Chuong, 1982; Hirn *et al.*, 1981). The molecule has

two forms; the adult (A) form and the embryonic (E) form. N-CAM undergoes conversion from the E form, with high sialic acid content, to the A form, with lesser amounts of this sugar, as development progresses. The A form of N-CAM contains significantly less sialic acid (Edelman and Chuong, 1982) and increases the rate of N-CAM binding (Hoffman and Edelman, 1983). This suggests that this binding may regulate pattern formation and connectivity in the adult nervous system (Edelman, 1983).

N-CAM is produced from a single gene and is a member of the immunoglobulin superfamily (Hemperly *et al.*, 1986b). This group of proteins shares a homology sequence of 100 amino acids connected by a disulfide bridge. The three polypeptide segments that make up the N-CAM molecule are encoded by different messengers and are produced by alternative splicing of a single gene. They differ mainly in their carboxyterminal part (Hemperly *et al.*, 1986a).

Approximately 98-99% of all brain N-CAM is membrane-associated. In muscle, however, a soluble form of N-CAM is synthesized as a primary translation product; its mRNA sequence distinguishes it from the membrane associated isoforms of N-CAM (Walsh, 1988). At present, little is known about this soluble N-CAM isoform.

Neurons adhere to other neurons by binding homophilically to the N-CAM on their respective surfaces (Hoffman and Edelman, 1983). In the embryo, N-CAM binding contributes to these processes: neural induction (Chuong, 1990),

neurite outgrowth (Rutishauser *et al.*, 1978; Rutishauser and Jessell, 1988; Chang *et al.*, 1987), formation of retinal layers (Buskirk *et al.*, 1980), nerve-muscle interactions (Grumet and Edelman, 1984), the ensheathment of axons (Rieger *et al.*, 1988) and the formation of synapses (Sanes, 1989). N-CAM is present from the blastoderm stage in chicken (Crossin *et al.*, 1985) and is present on most cells in the brain with a relatively uniform distribution (Persohn and Schachner, 1987). N-CAM is present on all neurons examined in both the central and peripheral nervous system (Thiery *et al.*, 1982; Mirsky *et al.*, 1986). In the peripheral nervous system, N-CAM is present on non-myelinating Schwann cells, nodes of Ranvier (Rieger *et al.*, 1986), and on satellite cells of sensory and sympathetic ganglia (Mirsky *et al.*, 1986).

Cell-cell binding occurs through a series of cell surface modulatory events that alter N-CAM affinity, prevalence, mobility and distribution on the surface (Cunningham *et al.*, 1987). These signals appear to be triggered by local signals produced by developing cells (Crossin *et al.*, 1985). Local signals between neurons and glia may regulate CAM expression in the spinal cord and nerve during regeneration, and that activity may regulate N-CAM expression in muscle (Daniloff *et al.*, 1986b).

In the brain, N-CAM has been shown to mediate calcium-independent neuron-neuron, astrocyte-neuron and astrocyte-astrocyte adhesion (Keilhauer *et al.*, 1985). Outside the nervous system, N-CAM mediates interactions between

several cell types, including nerve-muscle cell interactions (Rutishauser *et al.*, 1983). The adhesive activity has been demonstrated primarily through N-CAM antibody perturbation assays.

In mature tissues, N-CAM may be a local signal for reformation of nerve-muscle contacts following peripheral nerve injury (Daniloff *et al.*, 1986b; Rieger *et al.*, 1988; Doherty *et al.*, 1991). Local production of the embryonic (E) form of N-CAM, which is rich in polysialic acid, may modulate axon regeneration in an injured nerve (Edelman and Chuong, 1982; Daniloff *et al.*, 1986a; Friedlander *et al.*, 1985; Landmesser *et al.*, 1990).

The expression of N-CAM has been found to increase in response to nerve compression and transection, especially near an injury. Specifically it has been found to increase more in the proximal than the distal stump, where only the E form has been isolated. Immunohistological localization indicated that both Schwann cells and axons contain it (Daniloff *et al.*, 1986b).

One study found that non-myelinating Schwann cells formed slender processes which were N-CAM positive in the regenerating adult mouse sciatic nerve during and after degeneration of axons (the first 2-6 days) (Martini and Schachner, 1988). Only a few myelinating Schwann cells expressed N-CAM. Growth cones and regrowing axons expressed N-CAM at contact sites with fibroblast-like cells on the cut ends of nerve stumps. Regrowing small diameter axons were N-CAM positive where they contacted each other or contacted

Schwann cells. Schwann cells associated with degenerating myelin also expressed N-CAM (Martini and Schachner, 1988).

The presence of extracellular N-CAM near an injury suggests that its secretion could be an attractant for neurites or migrating Schwann cells. The localization of N-CAM on Schwann cells indicates that N-CAM could affect Schwann cell proliferation, a component of the peripheral nervous system's response to injury (Kruse *et al.*, 1984). N-CAM is also a component of the basal lamina (Rieger *et al.*, 1988) and shares a common epitope with myelin-associated glycoprotein (Madison *et al.*, 1987); this indicates that N-CAM may indirectly be involved in myelin formation.

Contributions of Trophic Factors

Trophic factors and their receptors are extracellular signals involved in the regulation of normal and injury-induced neurite outgrowth. It has been implied that peripheral effector organs produce limiting amounts of specific neurotrophic factors to ensure proper innervation of targets by appropriate neurites (Korsching and Thoenen, 1983). These factors are necessary to maintain the normal metabolic, functional and neurochemical state of the functional axon.

Nerve Growth Factor (NGF)

Nerve growth factor is the only defined molecule for which a chemotropic role has been postulated. *In vitro*, the growth cones of sensory neurons orient toward a source of NGF (Gundersen and Barrett, 1979). There are changes in

the quantities of NGF and its receptor in regenerating nerves (Heumann *et al.*, 1987). Very little NGF or NGF receptor is found in a normal peripheral nerve. However, if the nerve is cut or crushed, the level of both molecules and their respective mRNAs in the region distal to the injury increases enormously. The expression of NGF receptor by Schwann cells is probably controlled by axonal contact, and decreases when axonal contact is restored (Taniuchi *et al.*, 1986).

In one study: (1) the NGF receptor molecules were localized to the cell surface of Schwann cells forming bands of Bungner, (2) axonal regeneration in the distal portion of the sciatic nerve coincided temporally and spatially with a decrease in Schwann cell production of NGF receptor, (3) Schwann cell NGF receptors could be induced by axotomy of NGF-independent neurons, such as motoneurons and parasympathetic neurons, and (4) the presence of axon-Schwann cell contact was inversely related to expression of NGF receptors by Schwann cells (Taniuchi *et al.*, 1988). When Schwann cells are released from axonal contact, they express NGF receptors on their surface and also secrete NGF. This NGF receptor expression and NGF secretion by Schwann cells also occurs extensively in nerves undergoing active degeneration, and subsides again when nerve regeneration is completed. NGF is actually a potent modulator for increasing neurite sprouting from adult rat dorsal root ganglia (DRG) in culture (Taniuchi *et al.*, 1986).

Beta-nerve growth factor (ß-NGF) supports the differentiation, maturation and survival of sympathetic and primary sensory neurons (Thoenen and Barde, 1980; Raivich *et al.*, 1989). It is bound by NGF receptors, synthesized on the surface of neurites, internalized and then transported to the neural perikarya where it exerts its neurotrophic effects. Injury to a peripheral nerve results in a biphasic increase in the endoneural mRNA coding for ß-NGF, leading to an increase in the local synthesis of ß-NGF protein (Heumann *et al.*, 1987).

Peripheral axotomy also leads to a highly reproducible disappearance of neuronal ß-NGF receptors (Taniuchi *et al.*, 1986). Following transection or crush of the sciatic nerve, ß-NGF receptors disappear from the chromatolytic neuronal perikarya in the axotomized DRG by 6 days post-injury (Raivich and Kreutzberg, 1987). This decrease is greater after nerve transection than after a nerve crush. There is a concomitant decrease in retrograde axonal transport of endogenous NGF during sciatic nerve regeneration but the decrease stabilizes at a level 33% of normal control values (Raivich *et al.*, 1989). This decrease in retrograde transport corresponds to a decrease in ß-NGF in the proximal part of the regenerating sciatic nerve. With peripheral reinnervation there is a gradual increase in the axonal expression of ß-NGF receptors and ß-NGF retrograde transport. Both these values reach normal levels 30-40 days after the injury (Korsching and Thoenen, 1983).

Insulin-like Growth Factor-1 (IGF-1)

Insulin-like growth factor-1 has growth-stimulating activity and is believed to mediate some of the actions of growth hormone (Froesch *et al.*, 1985). IGF's promote neurite outgrowth in cultured sensory and sympathetic neurons (Recio-Pinto, 1986), spinal cord (Ishii *et al.*, 1989) and cloned human neuroblastoma cells (Recio-Pinto and Ishii, 1984, 1988). IGF-1 also stimulates proliferation and growth associated with nerve regeneration (Froesch *et al.*, 1985; Kanje *et al.*, 1989; Sjöberg and Kanje 1989; Hansson *et al.*, 1986).

Physiological concentrations of IGF-II can also support the long-term survival of peripheral neurons in culture (Recio-Pinto, 1986; Recio-Pinto and Ishii, 1988). One hypothesis is that IGF-1 produced by Schwann cells in the distal nerve segment is taken up by the regenerating nerve fibers. Internalized IGF-1 is then transferred to the nerve cell body by retrograde axonal transport. In the cell body, IGF-1 initiates or enhances the regenerative process, possibly by stimulating lipid and protein synthesis (Kanje *et al.*, 1990).

Growth Associated Protein (GAP 43)

B-50/GAP-43 is one of a small subset of cellular proteins selectively transported by a neuron to its terminals (Skene and Willard, 1981). Its enrichment in growth cones and its increased levels in developing or regenerating neurons suggest that it contributes to neurite growth (Verhaagen *et al.*, 1988). In adult rat DRG, crush lesions of the sciatic nerve result in a rapid expression

of B-50/GAP-43 mRNA followed by synthesis of B-50/GAP-43 protein and transport into the newly formed sprouts (Gispen *et al.*, 1990a). Normal neuromuscular junctions of the soleus muscle, one of the target muscles of the sciatic nerve, rarely contain GAP-43. During reinnervation following damage, neuromuscular junctions contain abundant amounts of GAP-43; these levels return to normal after completion of reinnervation (Gispen 1990a).

The precise function or mechanism of action of B-50/GAP-43 in developing, regenerating and adult peripheral and central nervous system neurons is still not clear. The recognition that the protein is a substrate for protein kinase C and that phosphorylation reduces its ability to bind calmodulin suggests that this protein is involved in a transmembrane signal transduction mechanism (Gispen *et al.*, 1990b). In the regenerating neuron, the axonal growth cone is enriched in B-50/GAP-43 and may need this protein either as a buffer of calmodulin or as a regulator in the polyphosphoinositide response to external signals that guide motility (Gispen *et al.*, 1990a).

Ciliary Neurotrophic Factor (CNTF)

The large quantities of ciliary neurotrophic factor present in the sciatic nerve of adult rats suggest the possibility of its function as a 'lesion' factor preventing motoneuron degeneration after nerve lesion (Manthorpe *et al.*, 1986; Stockli *et al.*, 1989). This suggestion is supported by the following observations: 1) transection of the facial nerve in adult rats, which contain almost exclusively

axons of motoneurons, results in chromatolysis in the motoneuron cell bodies; however, no degeneration of facial motoneurons occurs within 1 week (Tetzlaff *et al.*, 1988), 2) transection of the facial nerve in newborn animals results in a degeneration of all neurons (Sendtner *et al.*, 1990). The extent of degeneration decreases rapidly in the postnatal period and parallels the increase in levels of CNTF in the peripheral nerves (Stockli *et al.*, 1989). The fact that the local administration of CNTF in newborn animals can almost completely prevent the degeneration of the corresponding motoneuron cell bodies supports the validity of a causal relationship between the extent of degeneration and the levels of CNTF in lesioned nerves (Sendtner *et al.*, 1990).

OBJECTIVES OF THIS STUDY

Recent studies suggest cell interactions mediated by N-CAM may contribute to the regeneration of injured nerves and the reinnervation of target muscles (Daniloff *et al.*, 1986b; Rieger *et al.*, 1985; Rieger *et al.*, 1988). For example, anti-N-CAM antibodies have been used *in vivo* to perturb the formation of the retinotectal pathway in Xenopus (Fraser *et al.*, 1984) and to perturb retinal lamina formation in chick embryos (Buskirk *et al.*, 1980). The terminal Schwann cell was not found in the area of nerve-muscle contact when N-CAM antibodies were present (Grumet *et al.*, 1982). N-CAM antibodies also perturbed nerve-muscle interactions (Rieger *et al.*, 1985). The present study was undertaken to discover any underlying cell adhesion mechanisms that were blocked by attaching implants containing antibodies with anti-N-CAM like activity to injured nerves.

My primary goal was to describe any disruptions in the time course of *in vivo* muscle reinnervation that resulted when binding was blocked by monoclonal antibodies with anti-N-CAM like activity. These I will refer to henceforth as our antibodies. Tubes containing our antibodies were microsurgically attached to both stumps of transected sciatic (ischiatic) nerves. The entubulization model was based upon previously published observations of enhanced nerve regeneration with the implantation of inert tubes (Fields and Ellisman, 1986; Madison *et al.*,

1987; LeBeau *et al.*, 1988; Gibson *et al.*, 1989a,b), collagen (Bunge, 1980a), laminin (Madison *et al.*, 1987) or gangliosides (Mengs *et al.*, 1984).

Non-invasive muscle-evoked potentials were recorded to assess return of nerve function (Kline and Kahn, 1982). Enzyme-linked immunosorbent assays (ELISAs) were used to test sera for systemic immune responses to the implants and to estimate the amount of our antibody that remained in them.

Inert, plastic tubes containing our antibodies were microsurgically attached to the stumps of transected sciatic (ischiatic) nerves to span a gap of 4 mm. Control implants contained Thy-1 antibodies. In adult rodent brain Thy-1 occurs on almost every neuronal surface, and immunohistochemical labeling stains virtually all nervous tissue; peripheral nervous system axons have nearly twice the surface density of Thy-1 as central nervous system axons (Morris and Grosveld, 1989). Thy-1 antigen represents a model system for analyzing the properties of cell membrane structures shared between brain and lymphoid cell lineages (Naquet *et al.*, 1989). Both light and electron microscopic analyses were used to examine cellular interactions that occurred during sciatic nerve regrowth across the 4 mm nerve gaps.

METHODS

Antibody Production

Immunization

A monoclonal antibody against rat N-CAM was prepared using young adult female, Balb/C mice. The Louisiana State University centralized Tissue and Organ Culture Facility was utilized for these procedures. Initially mice were immunized intraperitoneally with 500 μl of saline containing 20 μg of purified rat N-CAM (obtained as a generous gift from the Edelman Laboratories, Rockefeller University, New York, NY) and 2 x 10^9 killed *Bordetella pertussis* organisms (Wako Chemicals, Dallas, TX). Subcutaneous booster immunizations for each mouse contained 10 μg live, dissociated embryonic rat brain cells (Hirn *et al.*, 1981) which were given on three occasions at 14 day intervals. The mice were killed three days after the final booster immunization and their spleens were recovered for the production of monoclonal antibodies (Oi and Herzenberg, 1980).

Isolation

The spleens from the immunized mice were aseptically harvested, placed in a petri dish containing complete Dulbecco's Modified Eagle's medium. This medium was injected into the spleen, causing it to swell and cells to be released. The tissue was teased apart with dissecting needles to release as many cells as possible. These cells were then centrifuged for 5 minutes at 200 x g. The

supernatant was removed from the pellet and the pellet was resuspended in Geys hemolytic medium. This mixture was allowed to stand at room temperature for exactly 5 minutes, then centrifuged for 5 minutes at 200 g. The cells were resuspended in 10 ml complete medium at room temperature. Hybridoma cells were selectively grown using standard techniques (Köhler and Milstein, 1975, 1976; Köhler *et al.*, 1976). The myeloma cell line (SP2/0-M14) was expanded. Spleen cells and partner cells were washed, harvested, and mixed.

Fusion

Cell fusion was performed at 37°C in the presence of polyethylene glycol (PEG). Cell count and viability of the myeloma cell were determined and a volume containing 2 x 10^7 cells was centrifuged at 200 x g for 5 minutes. Cell count and viability was also determined on the spleen cells. A volume of myeloma cell suspension was added to the spleen cell suspension (the spleen cell/myeloma cell ratio was 10:1). This cell mixture was centrifuged for 5 minutes at 200 g. The resulting pellet was harvested and plated into tissue culture plates. After incubation with hypoxanthine, aminopterin, and thymidine (HAT) medium and feeding over 2 weeks, the hybridomas were screened.

Two hundred sixty-four clones were isolated. By screening these clones, we determined that five had the capacity to recognize N-CAM as described by Edelman and Chuong (1982b) and by Chuong and Edelman (1985). An enzyme-linked immunosorbent assay (ELISA) procedure developed for the detection of

N-CAM antibodies (Chuong and Edelman, 1985) was one method used to screen the clones. The clones were also tested on Western blots against embryonic rat brain membrane extract (Appendix G).

Antibody Screening

N-CAM Enzyme-Linked Immunosorbent Assay Procedure

For each 96 well polyvinyl plate, 50 μl of N-CAM solution was applied to each well. This solution contained 1 mg rat N-CAM in 4.8 ml ELISA buffer (EPBS, Appendix B). Plates were incubated at 37°C until dry, and were fixed for 5 minutes with 100% methanol. After drying, plates were incubated at room temperature in EPBS plus 10% NGS for 1 hour to block non-specific binding. Then 1 μl of culture supernate from each clone was applied to each well in 50 μl EPBS (supplemented with 10 mg/ml bovine serum albumin). Plates were incubated overnight at 4°C. After washing three times in EPBS, plates were incubated in goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO) and horse anti-goat IgG conjugated to horseradish peroxidase (HRP) (Sigma Chemical Co.). Diaminobenzidine (Vector Laboratories, Inc., Burlingame, CA) was added to visualize the HRP. To estimate the affinity of each antibody to N-CAM, each plate was scanned colorimetrically in an ELISA reader (Dynatech Laboratories). Antibodies from four clones showed high positive affinity for the N-CAM antigen on their particular well (Appendix G). These were considered potential antibodies that recognize N-CAM and N-CAM-like proteins.

Determination of Recognized Protein Molecular Weights

Brains were dissected from 19 day rat fetuses and homogenized in calcium/magnesium-free (CMF) medium containing Aprotinin (200 units/ml; Sigma Chemical Co.). Plasma membranes were enriched by a one-step sucrose-gradient. Material from the surface interface was extracted with PBS containing 0.5% Nonidet P-40 and 1 mM EDTA. Aliquots (50 μl) of material in solution (containing 100 μg protein as determined by the BCA* protein assay) were resolved on 8.5% SDS polyacrylamide gels (Laemmli, 1970). Gel lanes were transferred to nitrocellulose membranes (Towbin *et al.*, 1979) and cut into individual vertical strips. Individual nitrocellulose strips were then incubated for 4 hours with the radiolabelled antibodies (3 μg/ml PBS-BSA) and washed three times. After the Kodak SB-5 X-Ray film that had been aligned with the gel at -70° for 1 month was developed and fixed, radiolabelled proteins were visualized (Friedlander *et al.*, 1985).

Antibody Isotyping

The clones were isotyped using a commercial kit (Sangstat Co., ISOSTAT Ab Kit, Palo Alto, CA). Instructions included with the kit were followed exactly.

Antibody Purification

Anion Exchange Chromatography Purification

Anion Exchange Chromatography Purification (Fahey and Terry, 1979; Sober and Peterson, 1958). Pre-swollen anion exchange DE52 cellulose (Whatman Specialty Products Inc., Fairfield, NJ) was used to purify the cell culture supernatant. About 15-30 ml of buffer are used for every dry gram of cellulose initially taken, or about 6 ml/g of wet ion exchanger. Two hundred grams of preswollen DE52 cellulose was stirred in 500 mls of 10X 0.175 M $NaPO_4$ for 13 minutes. The buffer was decanted off and replaced with 1X 0.175 M $NaPO_4$, stirred and allowed to settle. The slurry was allowed to settle and the supernatant was decanted. This step was repeated 3 times and the slurry was allowed to settle. The pH of the buffer/ion exchanger slurry was adjusted to 8.6 with 1N NaOH while stirring. This stirred slurry was then swirled and poured into a C26/40 column (Pharmacia, LKB; Catalog No. 19-5201-01) and was allowed to pack and fill the column.

The cell culture supernatant was dialyzed for 24 hours against the 0.175 M $NaPO_4$ buffer, pH 8.6 for 48 hours with changes 4 times daily. This mixture was then loaded onto the column at a flow rate of 1.0 ml/min. The elution of the sample was performed immediately using 1X 0.175 M $NaPO_4$ pH 8.6 followed by 1X 0.175 M $NaPO_4$ with 0.05 M NaCl added. Absorbance at 280 nm was measured and total protein was determined by the Pierce bicinchoninic

acid (BCA*) protein assay (Pierce, Rockford, IL; catalog #23220/23225, batch 900403081) (Redinbaugh and Turley, 1986); the presence of IgG was confirmed by gel electrophoresis.

Protein-A Purification

Protein-A Purification (Ey *et al.*, 1978; Lindmark *et al.*, 1983, Richman *et al.*, 1982) - To attempt Protein-A purification of our monoclonal antibody, a HiTrap™ Protein A Sepharose High Performance affinity column from Pharmacia LKB was used. This was a prepacked, ready-to-use, disposable column for purification and isolation of monoclonal and polyclonal IgG from ascites, serum and cell culture supernatants. The cell culture supernatant was centrifuged at 10,000 x g for 10 minutes and filtered (0.22 μm), then dialyzed against the start buffer (50 mM Tris buffer, pH 8.6) for 24 hours with 4 changes of buffer. The column was washed with 5 volumes of start buffer; the sample was applied to the column and allowed to flow through. The column was washed with 10 volumes of start buffer before elution with 0.1 M citric acid, pH 3.0. Aliquots (1 ml) were collected into tubes containing 1/10 volume of 1.0 M Tris-HCL neutralization buffer, pH 9.0. Absorbance at 280 nm was measured and total protein was determined by the Pierce bicinchoninic acid (BCA*) protein assay (Pierce, Rockford, IL; catalog #23220/23225, batch 900403081) (Redinbaugh and Turley, 1986); and the presence of IgG was confirmed by gel electrophoresis.

Protein-G Purification

Protein-G Purification (Akerstrom *et al.*, 1985; Björck and Kronvall, 1984) - MAb-Trap™ G from Pharmacia LKB Biotechnology, Inc., Piscataway, NJ was used to attempt protein-G purification of IgG. This column was designed for quick and effective purification of monoclonal and polyclonal IgG from ascites fluid, serum and cell culture supernatants. The cell culture supernatant was centrifuged at 10,000 x g for 10 minutes and filtered (0.22 μm). The column was equilibrated with 0.2 M sodium phosphate (pH 7.0) (binding buffer); the prepared sample was applied to the top frit and absorbed onto the gel. Unbound proteins were washed away with the same binding buffer. The bound IgG was eluted with a pH 2.7 elution buffer (1.0 M glycine-HCl) and collected into tubes containing 1/10th volume of a pH 9.0 neutralization buffer (1.0 M Tris-HCL). Absorbance at 280 nm was measured and total protein was confirmed by the Pierce bicinchoninic acid (BCA*) protein assay (Pierce, Rockford, IL; catalog #23220/23225, batch 900403081) (Redinbaugh and Turley, 1986); and the presence of IgG was confirmed by gel electrophoresis.

Avid AL™ Purification

Avid AL™ Purification (Khatter *et al.*, 1991; Füglistaller, 1989; Gassmann *et al.*, 1990) - Avid AL mini-columns from BioProbe International, Inc., (The Nest Group, Southborough, MA) were used to attempt the purification of the IgG from cell culture supernatant. Avid AL is a novel affinity gel

specifically designed to bind immunoglobulins from mammalian and avian species. It will also purify mouse monoclonal antibodies from hybridoma cell supernatant or ascites fluid (Khatter *et al.*, 1991; Füglistaller, 1989). All these immunoglobulins will bind to Avid AL under physiological saline conditions. The column was prepared by washing with 15 ml of a regeneration buffer (20 parts methanol, 80 parts 1% acetic acid) followed by equilibration by washing with 10 ml of a 0.01 M sodium phosphate buffer, pH 7.4 (PBS). The cell culture supernatant was centrifuged and filtered through a 0.22 μm filter. The pH of the hybridoma supernatant was then adjusted to a pH of 7.3. Three ml were applied to the column and allowed to flow through. The column was then washed with the PBS buffer until all the Phenol Red dye was removed. The sample was eluted with a pH 2.8, 0.05 M sodium acetate buffer containing 20% glycerol into tubes containing a 1/10 volume of 1 M Tris base buffer, pH 11.0. Absorbance at 280 nm was measured and total protein was confirmed by the Pierce bicinchoninic acid (BCA*) protein assay (Pierce, Rockford, IL; catalog #23220/23225, batch 900403081) (Redinbaugh and Turley, 1986); and the presence of IgG was confirmed by gel electrophoresis.

Antibody Characterization

Western Blot Procedure

1. Preparation of Rat Embryo Brain Membrane Extracts for Transfer to Nitrocellulose - Brains were removed from 19-day rat embryos and washed

with cold calcium-magnesium free (CMF) media. The tissue was homogenized in a Dounce homogenizer containing CMF supplemented with 200 units/ml of aprotinin (a protease inhibitor, Sigma Chemical Co.). The homogenate was centrifuged for 20 minutes at 15,000 rpm in a Beckman centrifuge (JA20 rotor) at 5°C. This step was repeated. The pellets were resuspended with 2.25 M sucrose in PBS buffer, 18 mls were placed in centrifuge tubes and each aliquot was overlaid with 8 mls of 0.8 M sucrose in PBS. These tubes were centrifuged for 1 hour at 100,000 rpm, 4°C in a Sorvall centrifuge (TD-65 rotor). The material at the interface between the two sucrose concentrations was collected and washed twice with PBS. The above membranes were suspended in an extraction buffer [10 mM Tris, 5 mM EDTA (pH 8.2) plus Nonidet P-40] and centrifuged for 40 minutes at 100,000 rpm, 4°C.

2. Sodium Dodecyl Sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as previously described (Laemmli, 1970). Gels were 7.5% acrylamide and samples of rat brain membrane extract were prepared by boiling for 3 minutes with mercaptoethanol. Gels were calibrated with the following protein standards: Rabbit skeletal muscle myosin (M_r = 200,000), *E. coli* ß-galactosidase (M_r = 130,000), Rabbit muscle phosphorylase b (M_r = 94,000), Bovine serum albumin (M_r = 68,000), and Hen egg white ovalbumin (M_r = 43,000) (Bio-Rad SDS-PAGE Molecular Weight Standards, High and Low Range, Richmond, CA).

3. Sample Transfer to Nitrocellulose - The electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets was performed according to the method of Towbin *et al.*, 1979 (Appendix B).

4. Autoradiographic Visualization of Proteins Recognized by Monoclonal Antibodies to rodent N-CAM - Nitrocellulose sheets were incubated for three hours in monoclonal antibody solution (0.4 mg antibody in saline) for three hours. This antibody with anti-N-CAM like activity was semi-purified from cell culture supernatant as described (Greenberg and Edelman, 1983). Proteins recognized by the antibody were visualized after lanes were incubated with goat anti-mouse IgG (0.4 mg/ml in saline; Sigma Chemical Co.) and horse anti-goat IgG conjugated to [^{3}H]-thymidine (Dupont Co., NEN Research Products, Boston, MA). Gels were treated with sodium salicylate (Chamberlain, 1979) for autoradiography. Gel blots were dried and exposed at -70°C (one month) to Kodak SB-5 x-ray film. Developed films indicated the molecular weights of proteins recognized specifically by this antibody. Control lanes, not incubated in any primary monoclonal antibodies were negative.

Retinal Perturbation Assay

For this assay, retinal fragments from 6 day old chick embryos were dissected from the region surrounding the choroid fissure; these were positioned with their vitreous side down on 1/4 of a 2 cm Millipore filter, 1.2 μm pore size, that had been wetted with medium and placed on a stainless steel grid in a tissue

culture dish. The medium was Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% volume of heat-inactivated fetal calf serum (LSU School of Veterinary Medicine Tissue Culture Facility). The purified IgG from cell culture supernatants of the clones 3D12A, 3B1A and a commercial antibody (Rat anti-Neural Cell Adhesion Molecule Monoclonal Antibody, Chemicon International, Inc., Temecula, CA; catalog # MAB310, Rat IgG2a) were dissolved in tissue culture medium to a concentration of 1 mg/ml. All cultures were carried out for 3 days at 37°C in an atmosphere of 5% CO_2, and the retinae were then fixed in 2.5% formaldehyde/0.2% glutaraldehyde in PBS for paraffin embedding.

Implant Preparation

Sterile tubes, 6 mm in length, were prepared of inert silastic (inner diameter 0.78 mm, outer diameter 1.25 mm; Dow Corning). For experimental animals, each tube contained 2.5 μg monoclonal antibody (3B1A) mixed with 20 μl of bovine collagen gel (Collagen Corp, Palo Alto, CA). Control animals received tubes containing 20 μl of collagen gel with 2.5 μg of monoclonal antibody to Thy-1 protein (Miles Corp), a neuronal surface protein (Campbell *et al.*, 1981; Letarte, 1984).

Surgery and Survival Times

Young adult female Sprague-Dawley rats weighing approximately 200 gm were anesthetized by intraperitoneal injection of chloral hydrate (8%; 0.33 ml/kg

body weight). One sciatic nerve was exposed from the sciatic notch to the tibial-peroneal bifurcation and transected at mid-thigh level. The proximal and distal stumps were attached to the ends of the 6 mm silastic implant with 9.0 silk suture (Ethicon Inc., Somerville, N.J.). Implants spanned a gap of 4 mm between the two stumps. The sciatic compartment was closed with 3.0 gut suture (Ethicon Inc.) and the skin was closed with 3.0 nylon (Ethicon Inc.). The contralateral limb was not manipulated, it served as the normal control for each animal.

Tubes were implanted into 34 rats. Twenty-five animals were used for physiological assessment and necropsy at 10 (n=7), 20 (n=6), 30 (n=6), or 60 (n=6) days of survival. These same animals from 10, 30 and 60 days were used for the morphology part of this study. The survival period of 10 days was chosen because it was found that chromatolysis, a product of Wallerian degeneration, was complete at this time (Ramon y Cajal, 1928b). Thirty days was chosen because one would be able to quantitate regeneration by histology to determine advance of neurites across a gap (Ramon y Cajal, 1928b); sixty days was chosen because the reformation of myelin should be well established (Seilheimer *et al.*, 1989; Wood *et al.*, 1990).

Nine were used to assess the levels of antibody (3D12A) available in the implants at 7 (n=3), 14 (n=3) or 28 (n=3) days of survival. The antibody diminution was evaluated at 7 days because it has been found that at 10 days after nerve transection, N-CAM levels were increased relative to control nerves

(Daniloff *et al.*, 1986b); at 14 days because in chickens, a large increase in N-CAM was observed after 10 days in extracellular connective tissues (Daniloff *et al.*, 1986a); and at 28 days because it was found that at 20 days after nerve transection, Schwann cells along the bands of Bungner expressed only low levels of N-CAM (Daniloff *et al.*, 1986b).

Recovery of Function

Physiologic assessment of recovery of nerve function was performed with a computer-based electrodiagnostic system (Compact Four, Nicolet Biomedical Instruments Co., Madison, WI). Electromyographic (EMG) recordings were made of gastrocnemius muscle contractions in response to transcutaneous stimulation of the sciatic nerve (Kline and Kahn, 1982). Sciatic nerves were activated when a bipolar stimulator was applied with conductive electrode gel to the skin over the nerve near the dorsomedial aspect of the femur proximal to the nerve lesion. Supramaximal stimuli (4-12 mA) of 100 μsec duration were applied at a rate of 2.1/sec. Muscle responses were recorded within a 10msec time window using a band width of 5-1,500 Hz. A concentric bipolar EMG electrode was placed in the belly of the gastrocnemius muscle and a ground electrode was placed subcutaneously between the stimulating and recording electrodes. Responses to trains of 50 stimuli were averaged twice to ensure repeatability; the two averages were then combined. Thus, each data point

represents a computer average of 100 responses. Measurements consisted of the latency to the initiation of the responses after the stimulus was applied.

Estimation of Antibody Remaining

A Non-Competitive ELISA (Weir, 1949) (Figure 1) was performed to estimate the amounts of the original murine IgG (3D12A) that remained in the implanted tubes 7, 14, and 28 days after surgery. A standard curve of antibody concentration was established by coating the bottom of each well of a microtiter plate with 50 μl of 1 mg/ml solution of affinity-purified rat IgG (Sigma Chemical Co.). One of twelve concentrations of mouse anti-rat IgG (Sigma Chemical Co.), which ranged equally from 0.1 through 1.2 mg protein, was added to individual microtiter wells and dried. Bound anti-rat IgG was visualized with orthophenaline diamine (OPD) after incubation with goat anti-mouse IgG conjugated with horseradish peroxidase (Vector, Inc). The optical density of each well was read in an ELISA reader (Dynatech MR700). Each concentration point was run six times, averaged, and plotted to produce a linear standard curve.

Contents of the implanted tubes in three animals per group were removed and microhomogenized immediately in 90 μl of sodium phosphate buffer in Dounce glass microhomogenizers. Triplicate samples (30 μl) of each homogenate were added to IgG-coated microtiter plates and incubated for 4 hours at 4°C. After incubation in peroxidase-conjugated antibodies, each well was visualized

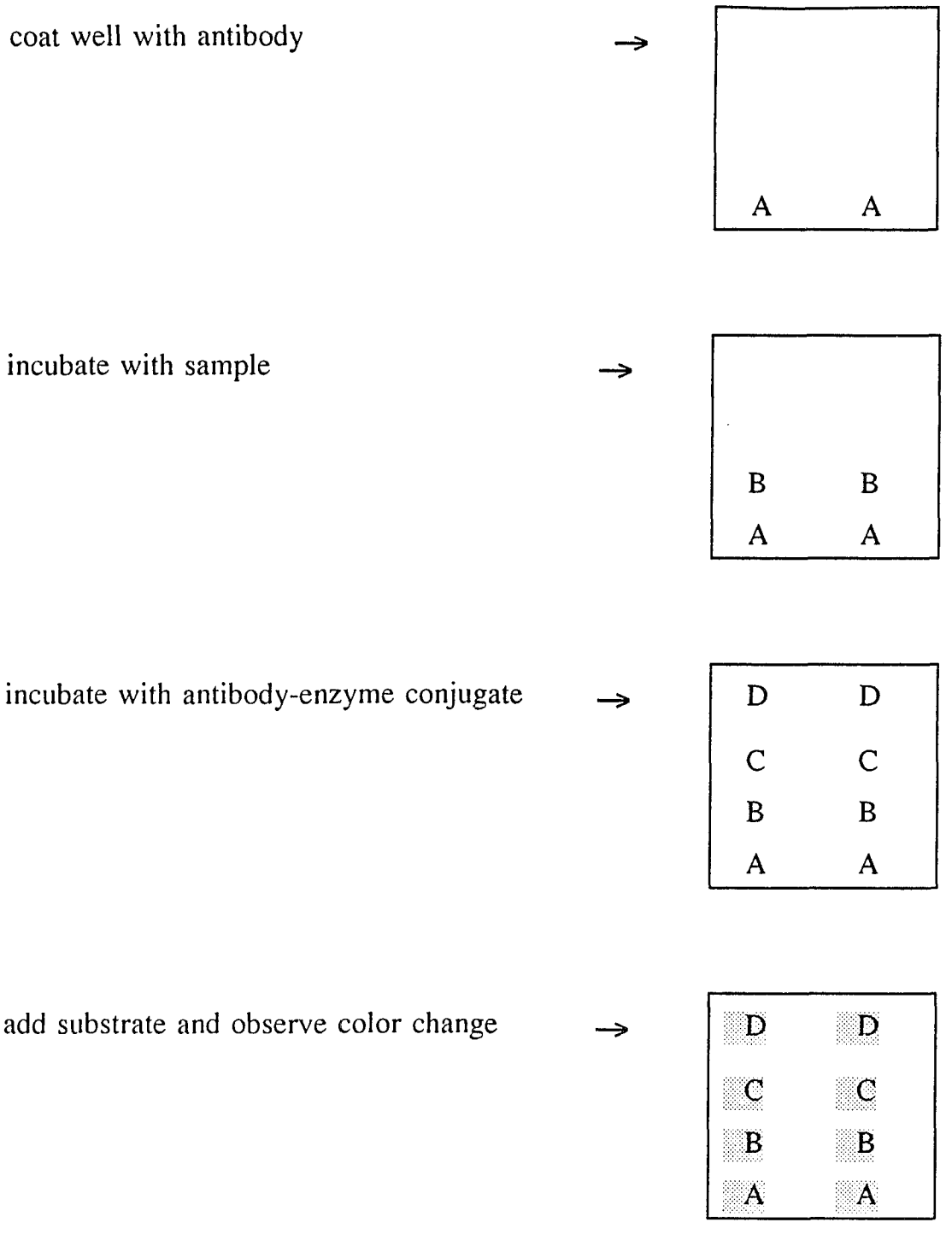

Figure 1: **ELISA for estimation of antibody remaining.** Antibody-sandwich ELISA (non-competitive assay) to detect murine IgG remaining in the implanted tubes. A = rat IgG, B = mouse α rat IgG (our antibody), C = goat α mouse IgG, D = HRP.

colorimetrically as described for the standards. To estimate the amount of mouse anti-rat N-CAM in a given animal, optical densities of triplicate samples were averaged.

Systemic Immune Responses to the Implants

Non-competitive ELISAs for antibody detection (Maggio, 1981; Kurstak, 1986) were performed on sera to determine whether systemic responses were mounted against either of the two components of the implants: bovine collagen and murine IgG (Figure 2). For these assays either 2.5 μg mouse IgG (Sigma Chemical Co.) or 20 μl of collagen gel (Collagen Corp., Palo Alto, CA) was added to microtiter plates. Serum was collected by caudal tail venipuncture from all rats on the following days after surgery: pre-treatment (0) and 3, 7, 10, 14, 21, and 28. Thirty microliters of each serum sample were tested in each well. Bound rat anti-mouse or rat anti-bovine IgG was visualized with orthophenaline diamine (OPD) after incubation with goat anti-rat IgG conjugated with horseradish peroxidase (Vector, Inc). The optical density of each well was read in an ELISA reader (Dynatech MR700). Each concentration point was run six times, averaged, and plotted to produce a linear standard curve. The limits of detection of this assay are 1 to 10 ng of protein.

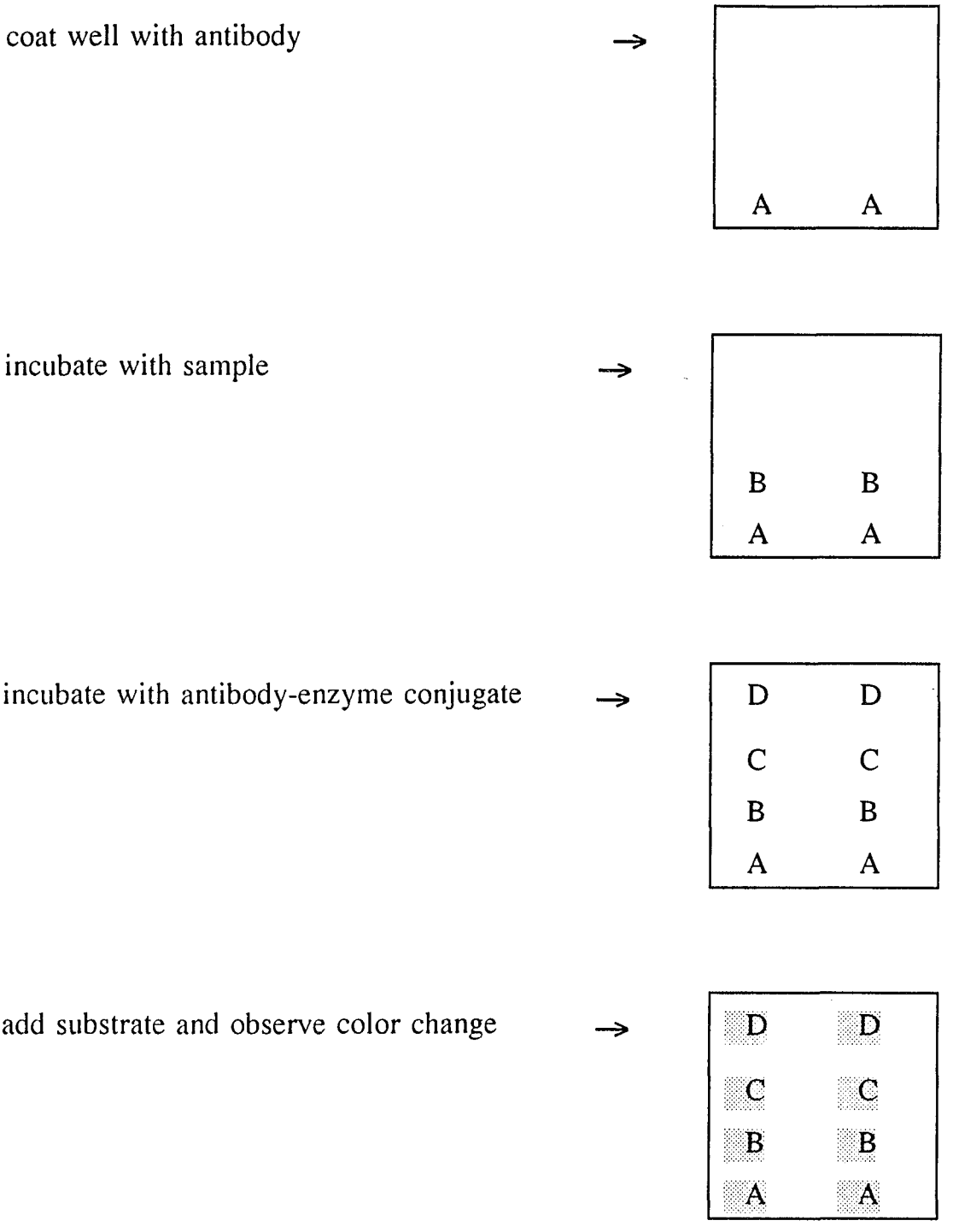

Figure 2: **ELISA for systemic immune response.** Antibody-sandwich ELISA (non-competitive assay) to detect serum immune response in operated animals. A = mouse IgG or bovine collagen gel, B = serum (rat α mouse IgG/rat α bovine IgG), C = goat α rat IgG, D = HRP.

Morphological Analyses

H & E Staining

At the three survival times, animals were deeply anesthetized and transcardially perfused for 15 minutes with a fixative (2.5% formaldehyde/0.2% glutaraldehyde in phosphate buffered saline (PBS)) for light microscopy as described previously (Daniloff *et al.*, 1986a). The portion of each sciatic nerve containing the implant was excised, removed from the tube and post-fixed for one hour in the same fixative. Fixed nerves were then incubated for 2 hours in 0.1 M glycine/PBS followed by an overnight incubation in 30% sucrose in PBS. Samples were immersed in embedding compound (OCT, Miles Scientific, IL) and quick-frozen in preparation for sectioning. Frozen sections (10 μm) of the distal half of the nerve implant were taken on a cryostat (Riechert-Jung, 2800 Frigocut), applied to glass slides, dried and stored in a dessicator at 4°C in preparation for antibody labeling. Several slides from the day 10 survival period were stained with hematoxylin and eosin (Preece, 1972) to identify any cell-mediated immune response generated by either the implant or our antibodies to N-CAM.

Immunohistochemistry

For immunofluorescent double-labeling, sections were incubated in 10% normal goat serum (NGS) in PBS for 2 hours to eliminate non-specific binding. Sections were then incubated overnight at 22°C with polyclonal S100 antibodies

(1:100 in 10% NGS, Chemicon, Inc., Temecula, California) to label Schwann cells (Holton and Weston, 1982a,b; Politis *et al.*, 1982) and monoclonal neurofilament antibodies (1:5 in 10% NGS, Dako Corp., Carpenteria, CA) to label axons (Höfler *et al.*, 1985). Polyclonal antibodies were visualized with rhodamine-conjugated goat anti-rabbit IgG (1:100 in 10% NGS, Cappel Laboratories; Cochranville, PA). Monoclonal antibodies were visualized by sequential incubation in biotinylated horse anti-mouse IgG and avidin-fluorescein-conjugated goat anti-horse IgG (1:100 in 10% NGS, Cappel Laboratories). Sections were viewed and photographed with an upright Olympus (Vanox) photomicroscope equipped with epifluorescent capabilities and appropriate filters for fluorescein and rhodamine optics.

Electron Microscopy

For ultrastructural analyses, animals were deeply anesthetized and transcardially perfused with fixative (2% formaldehyde/1% glutaraldehyde in 0.1M sodium cacodylate buffer containing 0.01M calcium chloride). After washing with buffer (0.1M PBS), nerves were immersed in 1% osmium tetroxide/PBS for 1 hour at room temperature then washed. Osmicated tissues were dehydrated in serial dilutions of ethanol before embedding in Epon/Araldite resin. Thick (1 μm) and ultrathin (70 nm) sections were placed on grids and stained with uranyl acetate and lead citrate for contrast. Schwann cells were identified and photographed through a Zeiss 10 transmission electron microscope.

RESULTS

Antibody Purification

The results of attempts to purify the IgG_{2b} with affinity chromatography using Protein G, Protein A and Avid A/L are shown in Table 1:

Table 1

Summary of Antibody Purification by Affinity Chromatography.

	Protein G	Protein A	Avid A/L
Initial Volume** (*absorbance units)	7.329	6.750	7.467
first wash*** (*absorbance units)	2.723	3.360	3.230
% recovery****	37.2	49.8	43.3
% bound to column	62.8	50.2	56.7
elution buffer	glycine-HCL pH 2.7, 1.0M	citric acid pH 3.5, 0.1M	sodium acetate pH 2.8, 0.05M
elution*** (absorbance units)	0.078	0.062	0.008
% recovery*****	1.1	0.9	0.1
% remaining on column	61.0	48.4	56.6

absorbance unit = (absorbance @ 280 nm)(volume)
**volume = 3 ml
***volume = 1.5ml
****% recovery = (first wash)÷(initial volume)
*****% recovery = (elution)÷(initial volume)

These results indicate that using Protein G for purification allowed 62.8% binding of the cell culture supernatant protein to the Protein G. The remainder of the prepared mixture flowed through the column without any binding. Of the bound IgG eluted under the manufacturer's recommended condition (1.0 M glycine-HCl, pH 2.7), 1.1% was eluted.

Of the applied culture supernatant proteins, 50.2% bound to the Protein A column; again, essentially none (0.9%) of the bound IgG was eluted under the manufacturer's recommended condition (0.1 M citric acid, pH 3.5). In retrospect it may be that IgG bound to the column was not eluted under the mild conditions prescribed by the manufacturer.

A similar finding was noted with the Avid A/L fast flow column. Of the applied cell culture supernatant protein, 56.7% bound to the beads while only 0.1% was eluted under the conditions 0.05M sodium acetate, pH 2.8. In all cases the eluted material (based on absorbance at 280 nm) was found to be IgG by gel electrophoresis. The proteins after denaturation with mercaptoethanol migrated on 7.5% SDS-PAGE as a discreet band at 66,000 indicating the presence of IgG (data not shown).

Anion Exchange Chromatography

IgG was ultimately purified by anion exchange chromatography using DE52 cellulose. Fractions eluted were assayed first by absorbance at 280 nm and then by gel electrophoresis. The protein after denaturation with

mercaptoethanol migrated on 7.5% SDS-PAGE at 66,000 indicating the presence of IgG (Hardy, 1986) (data not shown).

Antibody Characterization

Antibody Isotyping

Three clones were isotyped using the ISOSTAT Ab Kit and the following results were found: 3B1A was found to be IgG_{2a}, 3B1 was found to be IgM, and 3D12a was determined to be IgG_{2b}. The clone 3B1 proved to be very difficult to grow with viability around 20% in spite of environmental manipulation; therefore it was not used in this study. Purified supernatant from the 3B1A clone was used for the morphology and physiology portion of this experiment. As a result of accidental loss, we were not able to use this clone in additional portions of this study. The clone 3D12A was a very viable, healthy clone which was used for the measurement of systemic immune response and for the measurement of antibody remaining in the tubes.

Immunoblot Procedures

The 3D12A anti-N-CAM like antibody did not blot well on a Western blot against embryonic or adult brain membrane extract. The 3B1A clone did not blot at all. This has been reported previously (Chuong *et al.*, 1982; Chuong *et al.*, 1985; Hoffman and Edelman, 1983) for N-CAM monoclonal antibodies. This might be due to the fact that *in vivo*, N-CAM is membrane bound and *in vitro*

(with denaturation), the conformation change in the molecule that occurs may result in a decrease in the amount of binding sites available to the antibody.

Determination of the Molecular Weights Recognized by the Antibodies

Radiographic analyses were performed on brain membrane proteins resolved by

8.5% SDS-PAGE. Results showed that the radiolabelled antibodies indirectly identified a series of proteins very similar or identical to the embryonic form of N-CAM. The basic protein backbone of N-CAM proteins migrates at 180,000, 140,000 and 120,000 M_r on SDS-PAGE; the difference between the embryonic and adult forms is that the embryonic form contains approximately 10 times more polysialic acid than does the adult form (Edelman and Chuong, 1982b). The results of this analysis with our antibody are shown in Figure 3. It is possible that there were other proteins present which migrate with a pattern similar to N-CAM present, however, this was not determined.

Retinal Perturbation Assay

Tissues cultured in the presence of antibody (3D12A) showed disorder in the pattern of histological layers (Figure 4). In the control tissues, retinae developed in a normal manner. The commercial antibody also disrupted the pattern of development of the chicken retinae. Paraffin sections of retinae cultured in the presence of antibody revealed that the organization was clearly disrupted. The ganglion cells, normally arranged in an even layer along the

vitreous edge of the retina were much less organized and were found scattered throughout the inner plexiform layer. Also, the layers consisting of the bipolar and horizontal cells as well as the amacrine cells were not clearly distinguishable, suggesting that the distribution of specific cell types had been altered. This is the most widely accepted assay for demonstrating very specific immunologic perturbation by N-CAM antibodies (Buskirk *et al.*, 1980).

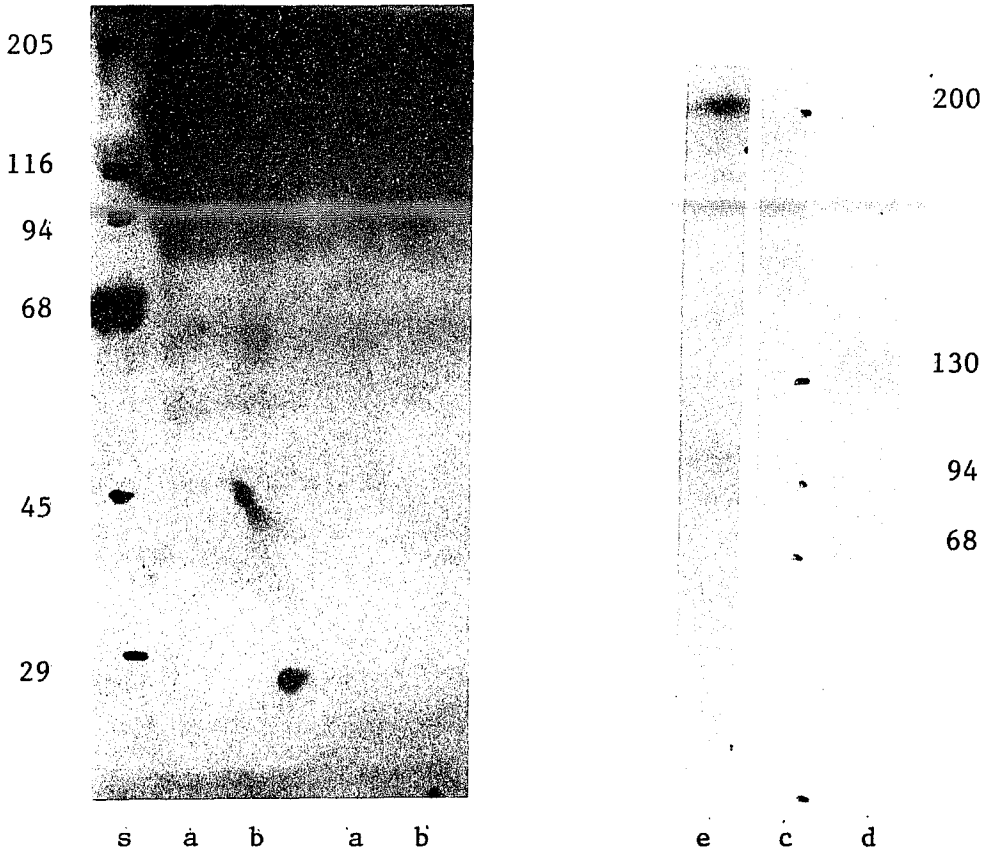

Figure 3: **Determination of Molecular Weights Recognized by Antibodies.** A. Lanes from 8.5% SDS polyacrylamide gel containing 100μg resolved cell membrane proteins. Proteins are labeled with MAb 3B1A (a), 3D12A (b), and visualized radiographically with iodinated second antibodies and x-ray film. This is very similar to that described for the embryonic from of N-CAM (Chuong *et al.*, 1982; Chuong and Edelman, 1985). B. Lanes from a Western blot of (c) standards, (d) embryonic brain membrane extract, and (e) embryonic brain membrane extract labeled with MAb 3D12A.

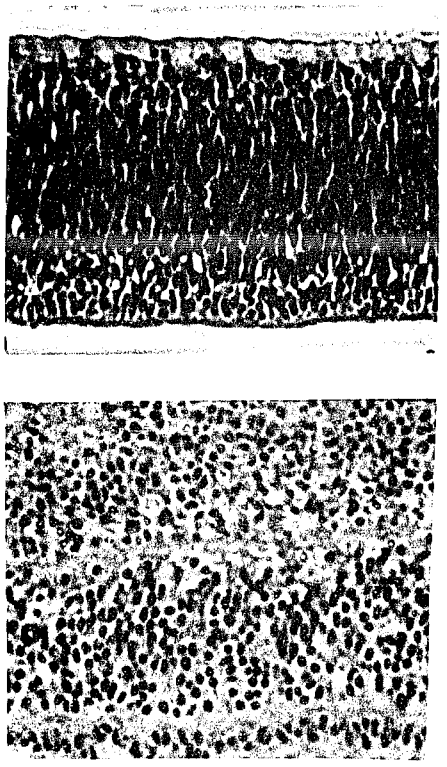

Figure 4: **Retinal Perturbation Assay**. A. Control day 9 retina. B. Day 9 retina cultured in the presence of the monoclonal antibody. The organization is clearly disrupted. The ganglion cells are much less organized and are found scattered throughout the inner plexiform layer. The layers consisting of the bipolar and horizontal cells are not clearly distinguishable.

Muscle-Evoked Potentials

The results of muscle-evoked potential recordings are presented in Table 2. In the experimental animals, muscle responses were absent at the 20 day survival point. The only tested time in which experimental responses differed significantly from controls was at 20 days. At this time there were no responses in the experimental animals; there were responses in the animals treated with antibodies to Thy-1. It has been previously shown that nerve regeneration in rats sustaining a sciatic nerve transection, is normally underway by 10 days (Daniloff *et al.*, 1986b). This was confirmed in the present study by the observation of muscle contractions in all of the control animals after 20 days. After 30 days, difference scores of experimental and control animals were similar.

Antibody Remaining in Implants

The average concentration of antibody remaining within the tubes dissipated over a 28 day period. Approximately 45% of the total remained after 7 days, 5% remained after 14 days, and after 28 days only trace amounts were detected. The percentage of antibody remaining in the tubes correlated negatively and significantly ($r=0.95$, $p<.01$) with the EMG response latency differences of the gastrocnemius muscles (Table 3). There was a response in this group of animals before the 20 day time point observed in the physiology portion of this study. Because Thy-1 had no apparent effect on the return of muscle function, its diminution over time was not examined.

Table 2

Latency (ms) between Stimulation and Initiation of Gastrocnemius Muscle Response (Clone 3B1A, IgG_{2b}).

	Survival (days)	Group	Treated leg	Normal leg	Difference scores	Group mean (SD) scores
I	20	Experi-mental	No response	2.56	Not calculated	Not calculated
			No response	2.24	Not calculated	
			No response	2.04	Not calculated	
			No response	2.60	Not calculated	
		Control	2.72	1.86	0.86	1.11 (0.25)
			3.20	2.08	1.12	
			2.84	1.48	1.36	
II	30	Experi-mental	2.52	1.40	1.12	1.26 (0.34)
			2.68	1.80	0.88	
			6.24	1.88	1.36	
			2.92	1.24	1.68	
		Control	1.88	0.24	1.64	1.24 (0.57)
			2.16	1.32	0.84	
III	60	Experi-mental	1.32	0.28	1.04	1.26 (0.52)
			3.92	3.28	0.64	
			1.96	0.36	1.61	
			3.52	1.76	1.76	
		Control	1.80	0.36	1.44	1.18 (0.37)
			3.72	2.80	0.92	

Note: Latency to muscle contractions (in milliseconds) for animals 20,30, and 60 days postsurgery. At 20 days the treated legs of all experimental animals failed to respond to stimulation, whereas all animals were significantly slower than controls after 20 days.

Table 3

Correlation between (B) Percentage of Normal Electromyographic Response Latencies and (C) Percentage of Original Antibody Remaining in the Implants (Clone 3D12A, IgG_{2b}).

(A) Survival time	(C) % Antibody remaining	Survival time	(B) % Normal EMG
7	34	10	0
7	56	10	0
14	6	20	83
14	4	20	83
28	0	30	98
28	0	30	99

Note: Corresponding survival times (A) are presented in the left-handed column. Pearson product moment correlation coefficient, $r = -0.95$ ($P < 0.003$, $r^2 = 0.91$).

Systemic Immune Response

Serum was tested 6 times from animals that were tail-bled from post-surgery day 3 to 28. There were no significant changes found in the levels of systemic rat antibody produced in reaction to the implanted murine IgG compared with pre-surgical serum levels (Table 4). A non-competitive ELISA was also run at the same time periods to test for the presence of systemic rat antibodies to bovine collagen. No significant alterations from pre-surgical levels were detected (Table 4). These data suggest that no systemic immune responses were mounted against either the murine IgG or the collagen component of the implants.

Measurement of systemic response to anti-Thy-1 was not done because these antibodies had no apparent effect on the return of muscle function.

Table 4

Immune Responses (in Optical Density Units) in Serum of Experimental Implant Recipients after Eight Survival Points: 0 (prebleed), 3,7,10,14,20, and 28 days.

		Survival day						
	Units	0	3	7	10	14	20	28
(A)	Mean	1162	839	897	1230	863	865	801
	(SD)	(289)	(453)	(264)	(263)	(230)	(230)	(234)
(B)	Mean	151	240	220	180	170	150	260
	(SD)	(141)	(190)	(200)	(180)	(170)	(180)	(150)

Note: Responses: (A) to anti-mouse IgG and (B) to bovine collagen.
Values are means and (standard deviation) of seven animals.

H & E Staining

As shown in Figure 5, there was no obvious cell-mediated immunity as a result of the implant or the antibodies to N-CAM. There is not more than the occasional granulocyte or macrophage found in the following 10 day sections.

Immunohistochemistry

Cross-sections of fixed distal nerves were taken after 10, 30 and 60 days post-surgery and stained with antibodies to visualize Schwann cells and axons. Abnormal and frequent gaps were observed in nerves receiving antibodies with anti-N-CAM like activity (Figure 6A). Few Schwann cells (Figure 6A') were observed and axons (Figure 6A") were not identified. In control nerves at

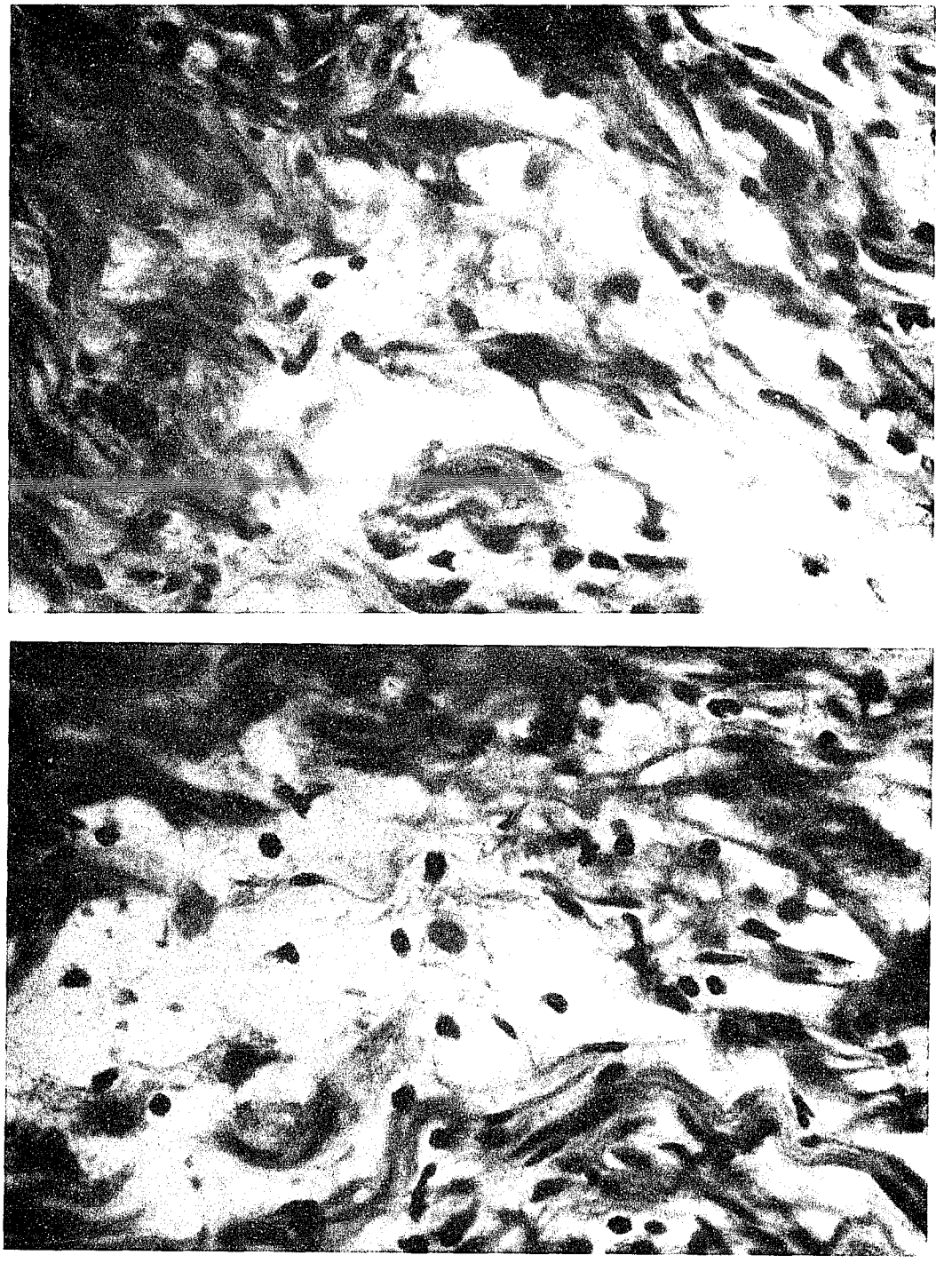

Figure 5: **H & E staining of cross-sections of 10-day distal nerve in the tube.** There was no obvious cell-mediated immunity as a result of the implant or the antibodies to N-CAM. Only the occasional macrophage is seen. (A) Control nerve. (B) Experimental nerve.

10 days of survival, as expected for severely injured nerves, gaps were present (Figure 6B). Schwann cells were sparse (Figure 6B'), and axons were not observed (Figure 6B"). These findings indicate a lack of reorganization for all groups at this time.

Nerves treated with antibodies with anti-N-CAM like activity maintained their lack of organization at the 30 day survival point (Figure 7A). Smaller and less frequent gaps were present when compared to the 10 day experimental nerves. Morphology of control nerves, as shown in the phase contrast photomicrograph (Figure 7B), reflected more advanced reorganization than that shown in experimental nerves. The lack of expected reorganization 30 days after nerve transection (Daniloff *et al.*, 1986b; Ramon y Cajal, 1928a,b) was a consistent finding noted only in experimental nerves.

Electron Microscopy

At this same time point, as depicted in the electron micrograph, abnormal gaps were frequently observed between apposing Schwann cells (Figure 8). Interactions between Schwann cells and axons were not noticeably perturbed.

After 60 days, nerves in both control and experimental nerve groups were more reorganized (Figure 9A,B) when compared with earlier survival points. The arrangement of Schwann cells (Figure 9A',B') and axons (Figure 9A",B") was more regular and compact than that in earlier surviving nerves. The appearance of the connective tissues surrounding the distal axons (Figure 10) suggested that myelin has been reformed around the reorganized fibers (Tanaka

and Webster, 1991). This reformation occurred after the time of antibody clearance from the implants.

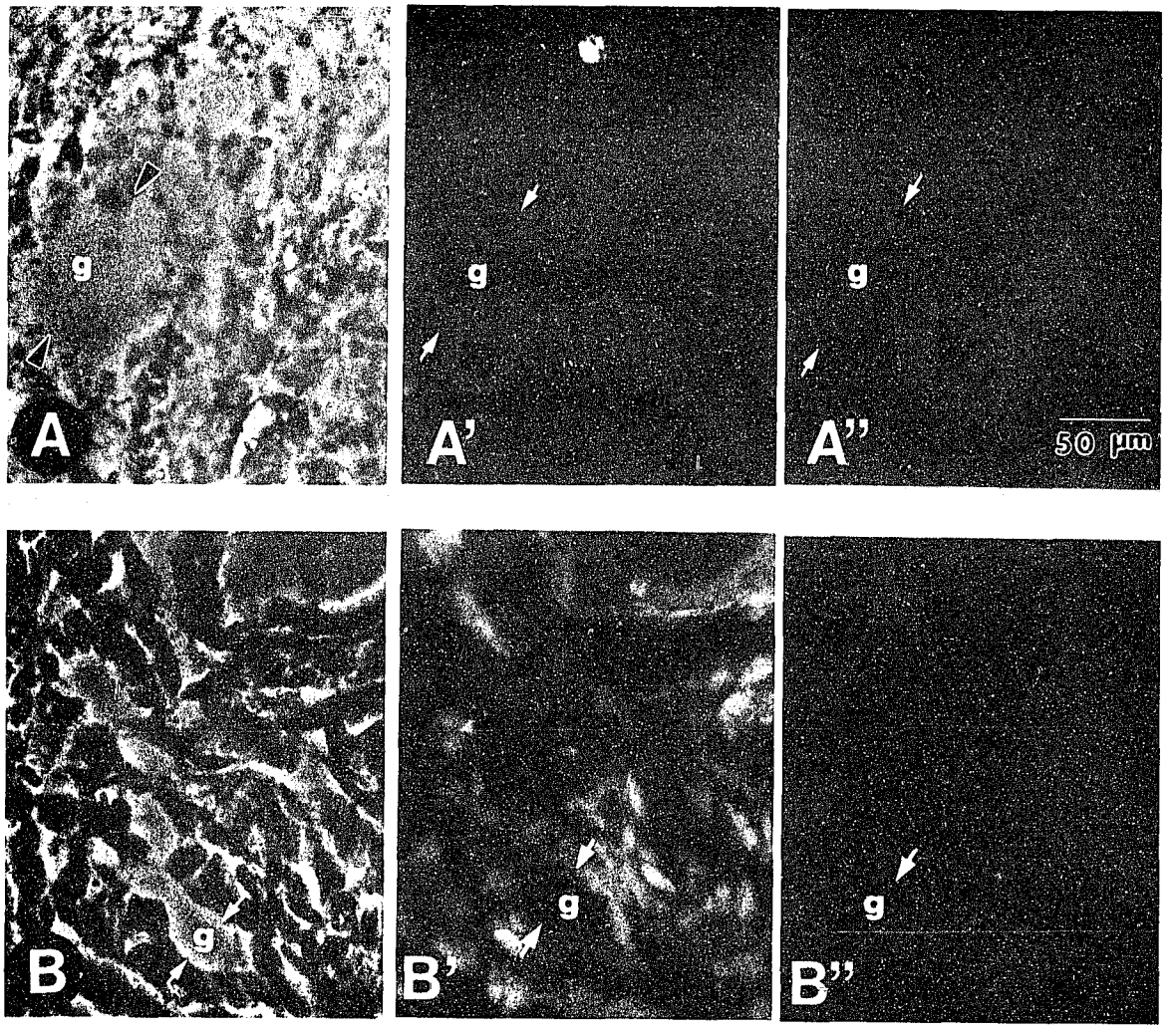

Figure 6: **Cross-section of distal nerves 10 days after surgery.** Phase contrast photomicrographs depict nerve morphology (A=experimental, B=control). Sections were double-labeled with polyclonal S100 (A',B') and monoclonal neurofilament antibodies (A",B"). In animals treated with experimental antibodies, gaps (g) in tissue were prevalend (A), some S100-positive Schwann cells (A') were identified although no identifiable neurofilament-stained nerves (A") were observed. Control nerve morphology (B) depicts disarray typical of transected nerves. S100-positive Schwann cells (B') were present in the absence of neurofilament-labeled regenerating axons (B"). Size bar = 50μm.

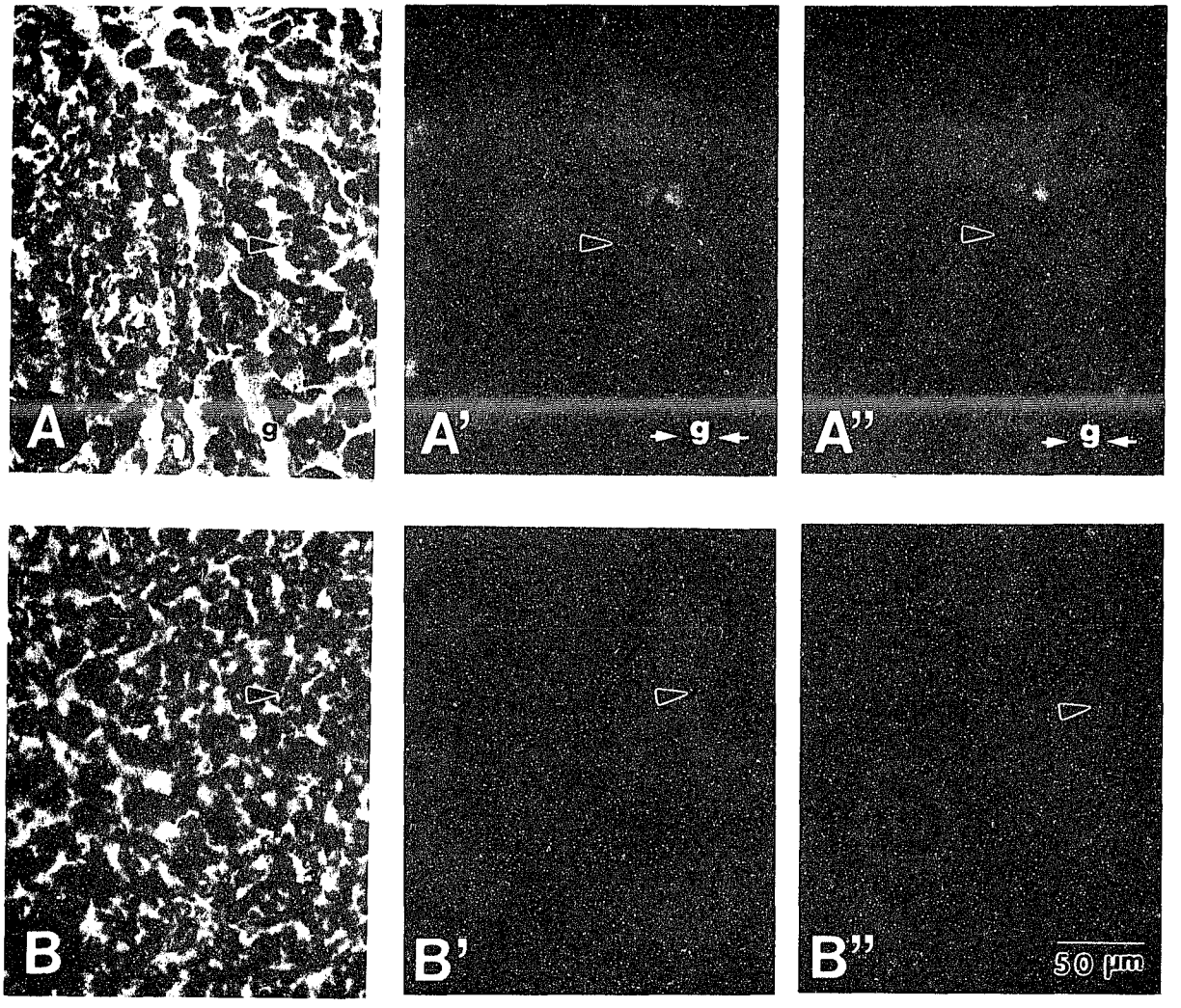

Figure 7: **Cross-section of distal nerves 30 days after surgery.** Phase contrast photomicrographs depict nerve morphology (A=experimental, B=control). Sections were double-labeled with polyclonal S100 (A',B') and monoclonal neurofilament antibodies (A",B"). Gaps (g) were still present in the experimental nerves (A), but were no longer present in control nerves (B). Axons (white arrows), labeled by neurofilament antibodies were identified in both control and experimental nerves at this time (A",B"). Size bar = 50μm.

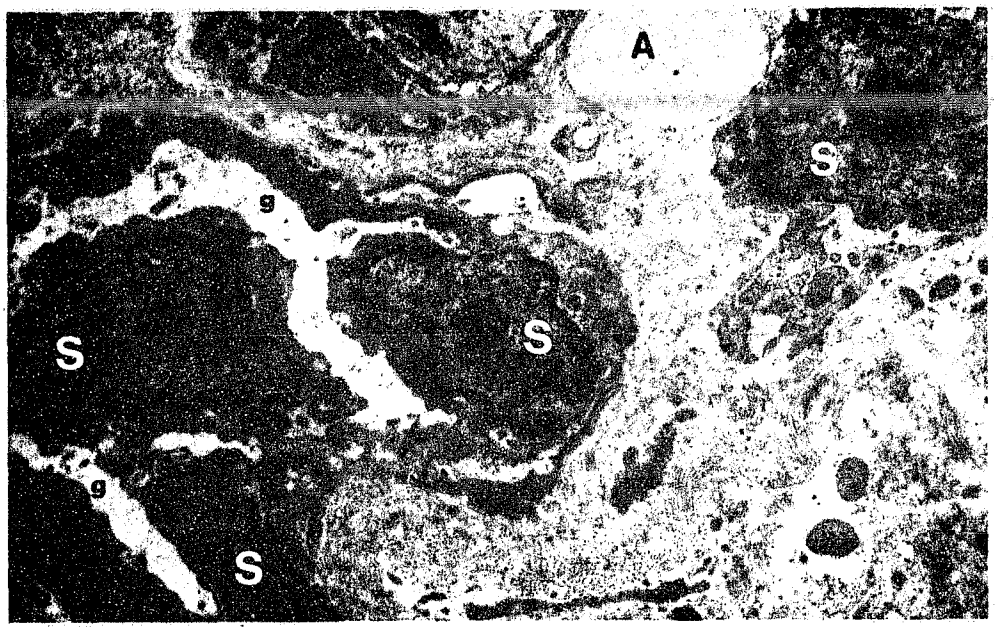

Figure 8: **Ultrastructural photomicrograph of distal experimental nerve 30 days after surgery.** Electron micrograph, transverse thin section (70 nm) of experimental distal nerve. Obvious gaps (g) between Schwann cells (s) were observed at this time point, however no apparent abnormalities between Schwann cells and axons (a) were noted.

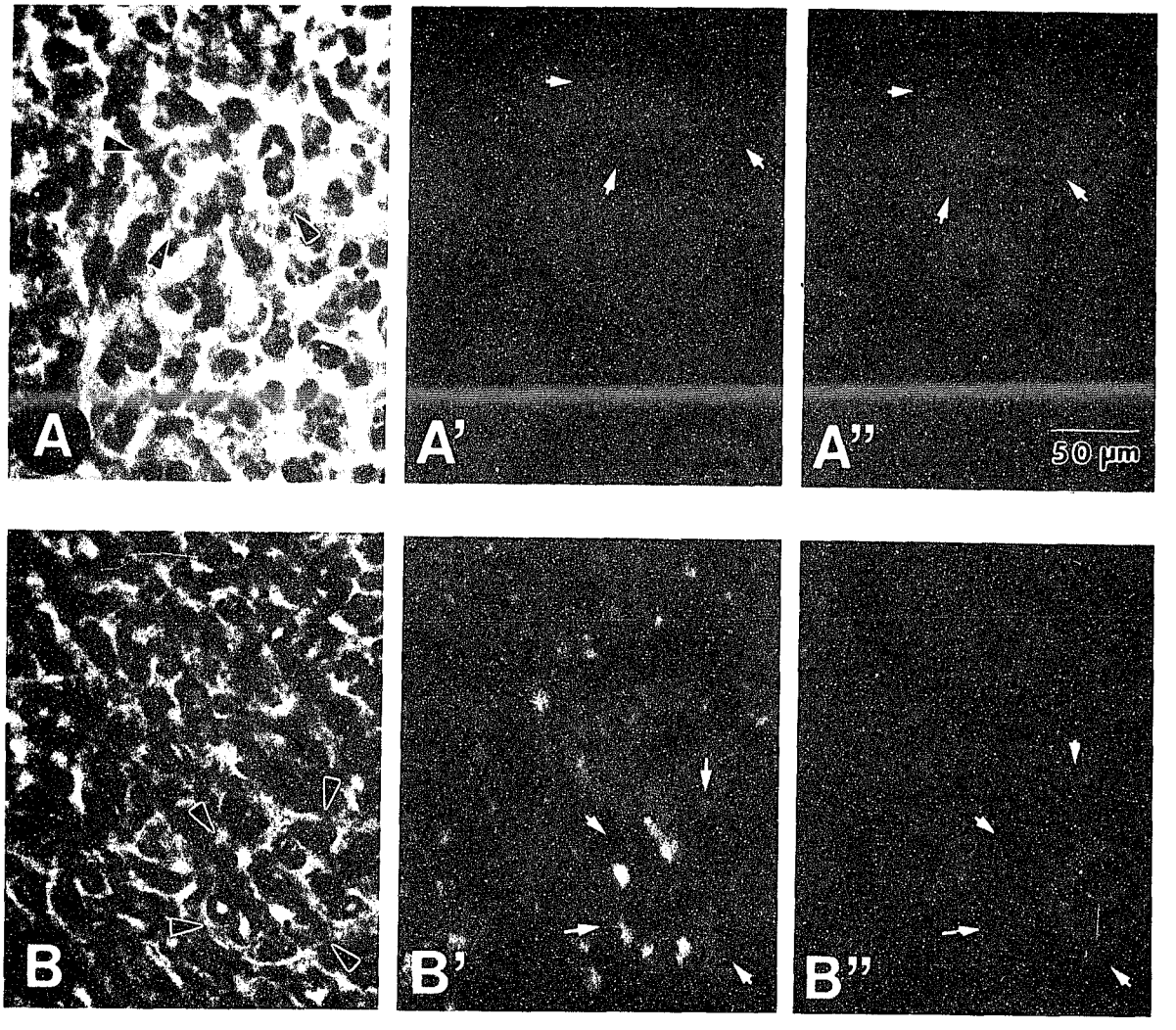

Figure 9: **Cross-section of distal nerves 60 days after surgery.** Phase contrast photomicrographs depict nerve morphology (A=experimental, B=control). Sections were double-labeled with polyclonal S100 (A',B') and monoclonal neurofilament antibodies (A",B"). Clusterina axons (arrows) occurred in both control and experimental nerves. Schwann cells (A',B') and neurofilament-labeled axons (A",B") were also present. Size bar = 50 μm.

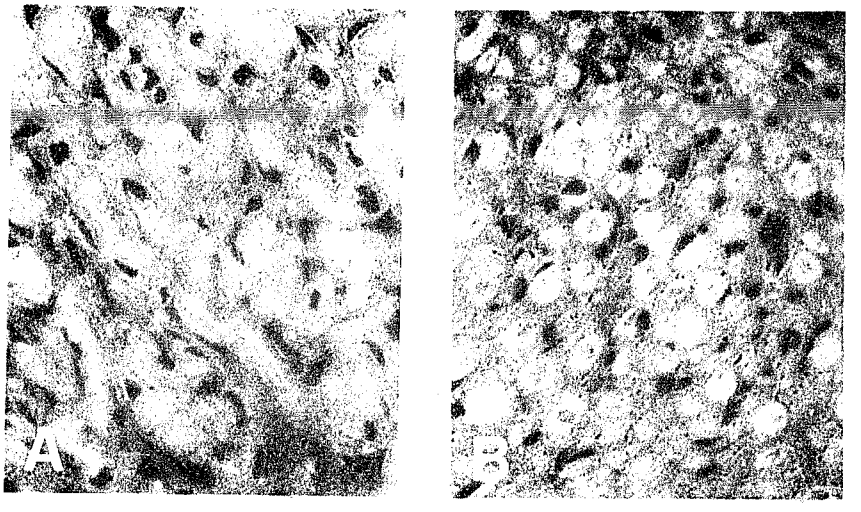

Figure 10: **Detailed analysis of nerve morphology at 60 days survival.** Photomicrographs of plastic embedded nerves (1 μm thick sections) depict morphological reorganization in experimental (A) and control (B) nerves at higher magnification.

DISCUSSION

Antibody Production - Use of *Bordatella pertussis* as an Adjuvant

Adjuvants are nonspecific stimulators of the immune response necessary to induce a strong antibody response to soluble antigens. Most adjuvants incorporate two components. One is a substance designed to form a deposit protecting the antigen from rapid catabolism. The two traditional methods of forming a deposit are to use mineral oils or aluminum hydroxide precipitates (Glenny *et al.*, 1926). The second component needed for an effective adjuvant is a substance that will stimulate the immune response nonspecifically. These substances act by raising the level of a large set of soluble peptide growth factors known as lymphokines. Lymphokines stimulate the activity of antigen-processing cells directly and cause a local inflammatory reaction at the site of injection. Heat-killed bacteria such as *Bordatella pertussis* or *Mycobacterium tuberculosis* are the most commonly used adjuvants. The immunomodulatory mediators of *B. pertussis* include a lipopolysaccharide component and the pertussis toxin (Dienes, 1936; Ellouz *et al.*, 1974).

Antibody Purification

Anion Exchange Chromatography

The major contaminant protein in ascites fluid, serum or ammonium sulfate precipitates, and tissue culture supernatant derived from antibody-containing fluids is albumin, which binds DE52 tightly under conditions of low-

to-moderate ionic strength. Antibody either fails to bind to DE52, in which case it elutes in the void volume as the column is loaded, or it binds loosely, and can be eluted with a gentle salt or pH gradient. Fractions eluted were assayed first by absorbance at 280 nm and then by SDS-PAGE (data not shown).

Protein A Purification

Protein A is produced by a selected strain of *Staphylococcus aureus*, is a functionally bivalent single polypeptide and has a molecular weight of 42,000. It has been proven to be useful in a variety of immunoassays because of its high affinity for immunoglobulins, its low level of nonspecific binding, and its ability to react with immunoglobulins of many different species (Kessler, 1975). The classical Fc-binding capacity of Protein A is restricted to IgG subclasses (Lindmark, *et al.*, 1983), except for other human classes of Ig which have been variably shown to bind to Protein A mediated by Fab structures (Björck and Kronvall, 1984). This alternative Fab-mediated Ig binding is of low avidity, and will show up when Protein A is present in excess in relation to IgG-Fc structures (Inganas, 1981).

Protein A consists of six different regions, five of which show strong, specific binding for the Fc-part of IgG of many mammalian IgG subclasses, leaving the antigen-binding sites free (Kessler, 1976). Immobilized Protein A can bind at least two molecules of IgG per molecule. When Protein A sites were provided in excess over IgG sites, virtually all IgG antibody molecules purified

from a hyperimmune rabbit antiserum against BSA bound firmly to the adsorbent (Kessler, 1976). Some monoclonal antibodies have a low affinity for Protein A, such as some mouse IgG_1 and human IgG_3 monoclonal antibodies (Larsson and Holmdahl, 1990).

Affinity chromatography on Protein A columns is widely used for the purification of monoclonal antibodies (Larsson and Holmdahl, 1990). The antibodies are normally applied to the column at neutral pH or at pH 8-9 and eluted at a lower pH. Application at a higher pH will increase the binding of some mouse antibodies to the column (Ey *et al.*, 1978). Mouse IgG is eluted from Protein A columns between pH 7.0 and 3.5 (Lindmark *et al.*, 1983), however a low pH will inactivate some monoclonal antibodies. In this project, only 50.2% of the IgG present in the ascites did indeed bind strongly to the Protein A. The capacity of this column was not exceeded, therefore the conditions for binding should have been optimized.

Individual monoclonal antibodies are found which do not conform to the general rules. Some hybrids which secrete antibodies give rise to partially active or inactive forms of the general form AA, AB, BB, where A and B are the two different (active and inactive) heavy chains which will be present in the ratio 1:2:1. Since different subclasses or microheterogeneity may be represented by the individual heavy chains, it follows that they may be resolved into three peaks or a broad Protein peak. The primary Protein A binding site on IgG is probably

present in the intact CH2-CH3 region of the heavy chains. If either chain of the pair is damaged or missing, then affinity will be reduced (Jack, 1990).

Conversely, if aggregates or polymers are present, then affinity may be increased. These situations are manifest by minor peaks eluting earlier or later than the main peak. Other binding sites in the IgG molecules are occasionally seen, though these are weaker and more variable in occurrence (Zola, 1990).

In retrospect, it appears that while our antibody bound to the column, it did not elute (% recovery = 1.1) with a 0.1 M citric acid buffer, pH 3.5. It has been shown in a study where PBS, pH 8.0 was used to wash the column following application of serum, no IgG_2 was found in the effluent even after prolonged washing. Elution of bound IgG with 0.58% acetic acid confirmed that the IgG_2 had been retained on the column (Ey *et al.*, 1978). These results indicated that binding of IgG_2 to Protein A could occur and that the association constant for this binding was extremely pH-dependent. In this referenced study, IgG_1 was eluted at pH 6.0, IgG_{2a} eluted at pH 5.0 while IgG_{2b} was not eluted until the buffer was at pH 3.0 - 4.0 (Ey *et al.*, 1978).

The reason for the low apparent recovery of IgG_{2b} in the present study is not known. We did not have evidence of non-binding of IgG to the column. Fractions were collected directly into tubes containing 1 M Tris-HCL buffer (pH 9.0) to immediately neutralize the eluate. Therefore, acid denaturation was probably not a factor. It is possible that some IgG_{2b} was lost through digestion

by contaminating protease(s), as mouse IgG_{2b} has been shown to be more sensitive than IgG_1 to proteolytic cleavage (Dessanayake and Hay, 1975).

Protein G Purification

Protein G-Sepharose chromatography offers a rapid method of purifying most subclasses of IgG. Protein G (Pharmacia, LKB) is a cell-surface protein of group G *Streptococci* and can be used to purify human, rat and mouse IgG. It is similar to Protein A in that it binds the Fc portion of the immunoglobulin. The capacity of Protein G is higher than that of Protein A. The absorbance at 280nm of 2.224 of the initial 3 ml sample indicated a protein concentration of 1.6 mg/ml. The capacity of the Pharmacia MAbTrap™ Fast Flow column was 6-7 mg/ml of gel. Since approximately 4.8 mg protein was applied to a 1 ml bed volume, the capacity of the column was not exceeded (80%). It was noted that approximately 62.8% of the applied protein bound to the column, however, after elution with 1.0M glycine-HCl, pH 2.7, 61.1% of the original material bound remained on the column.

Avid AL™ Purification

The binding capacity for IgG_{2b} from mouse for Avid AL is 5-7 mg IgG/ml of gel. Three mls of concentrated cell culture supernatant with an IgG concentration of 1.4 mg/ml (as determined by absorbance at 280 nm) were applied to this column; therefore approximately 4.2 mg of IgG was applied to a 1 ml column. The capacity of the column was at 70% which should have

allowed for maximum Fc binding. Approximately 56.7% of the IgG bound to the column, however, it did not elute under the conditions of 0.05 M sodium acetate buffer, pH 2.8 as recommended by the manufacturer of the kit. The elution fraction was collected at 280 nm and was determined to be IgG by gel electrophoresis. It is possible that the same variables as discussed in the previous two sections occurred and further studies as to possible elution methods are warranted.

Antibody Characterization

Determination of Molecular Weights

Gels have been widely used as the medium for immunoblot procedures. In this study, the radiolabeled anti-N-CAM like antibody recognized a broad band at MW 140,000 and minor bands at MW 180,000 and 120,000 when incubated with rat brain membrane extract on nitrocellulose. This pattern is similar to that of chicken N-CAM (Daniloff *et al.*, 1986b); this lead to the assumption that the antibody in question did have anti-N-CAM like activity.

Retinal Perturbation Assay

Anti-N-CAM antibodies alter the appearance of histotypic patterns in retinal cell aggregates maintained in culture for several days (Rutishauser *et al.*, 1978). It has also been found that the antibodies can disrupt histogenesis of the developing retina in organ culture (Buskirk *et al.*, 1980). In the chick, cell and plexiform layers of the central portion of the retina are formed between the sixth

and ninth days of embryonic life (Kahn, 1974). Retinae dissected from chicken embryos on day 6 and placed in organ culture for 3 days undergo histogenesis similar to that which occurs *in vivo*. Our results demonstrate that our monoclonal antibodies have anti-N-CAM like activity and disrupted the internal development of a tissue never subjected to mechanical dissociation. Because individual cells in the anti-N-CAM-treated retinae were morphologically similar to those in the control tisssues, it seems that the effects are not simply a consequence of changes in the developmental pathway of individual cells (Buskirk *et al.*, 1980). This conclusion was supported by the ability of the anti-N-CAM treated retinae to form some histological layers which, although altered, could be directly identified with layers in the normal retina (Buskirk *et al.*, 1980). Since the application of the monoclonal antibody used in this study disrupted the organization of the retina as shown in Figure 4, it can be assumed that our antibody had anti-N-CAM like activity, however, the possibility exists that our antibodies recognized other cell adhesion molecules and simply cross-reacted with N-CAM.

Use of Thy-1 Antibodies as Control

Thy-1 has been identified on almost every type of neuron and axon in the peripheral nervous system of the rat: sympathetic and parasympathetic ganglion cells, interneurons in sympathetic ganglia, preganglionic sympathetic axons, sensory ganglion cells and motor axons (Morris *et al.*, 1985). Thy-1 is a major

glycoprotein of the neuronal surface; it has been estimated that Thy-1 constitutes 2.5-7.5% of the surface protein of the following axons: myelinated CNS axons of the optic nerve, large myelinated motor axons of the hyposlossal nerve and the small, unmyelinated axons of the preganglionic sympathetic chain (Beech *et al.*, 1983). Thy-1 has not been found on glial cells in normal adult nervous tissue (Mirsky and Thompson, 1975). It has also been found that Thy-1 antibodies coated as a substrate on a culture dish promoted neurite outgrowth by Thy-1-positive neurons [retinal ganglion cells (Leiffer *et al.*, 1984) and Purkinje cells (Messer *et al.*, 1984)]. In the present study, antibodies to Thy-1 protein, which have affinity for the surface of cells derived from the brain in addition to the thymus (Campbell *et al*, 1981), were used in control tubes. Antibodies to Thy-1 were used as controls: (1) for steric hindrance and (2) because the protein has no known contribution to the growth or adhesion of nerves *in vivo* (Letarte, 1984).

Muscle Evoked Potentials

Electromyographic latencies were used as a non-invasive estimate of nerve patency. Our non-invasive recordings only approximated nerve conduction velocity, which can be correlated directly with the number of rapidly conducting nerve fibers and the degree of myelination of the nerve (Gibson *et al.*, 1989a). However, recordings of conduction velocity by direct exposure and stimulation of the nerves would have precluded the planned ultrastructural and morphological

examination of the tissues. The reproducibility of the latency recordings was the primary rationale for its use in these experiments.

The latency data (Table 2) showed that regeneration was significantly perturbed at 20 days after implantation. No muscle contractions were evoked in experimental animals but responses were evoked in all controls. This is a particularly relevant observation because it has previously been shown that active regeneration is present in rodents by 10 days (Daniloff *et al.*, 1986b). This lack of response in experimental nerves was no longer present at 30 days post-surgery. Although the average response for experimental animals was slower than that of controls at 30 days, the differences were not statistically significant. The validity of this analysis is enhanced because normal responses of contralateral (unoperated) legs were used as internal controls. There was no evidence of any experimental perturbation after 60 days.

Antibody Remaining in Implants

Perhaps a unique aspect of this investigation was the quantitation of N-CAM antibody levels remaining in the tubes during various stages of survival. Results indicated a progressive disappearance of the N-CAM antibody from seven days post-surgery, when approximately 45% remained, to twenty-eight days, when only trace amounts were detected. It is unclear why there were muscle responses in this group before 30 days. It is conceivable that this was due to individual animal variability. Another explanation may be that there was a

difference in antibody activity between clones 3B1A used in the physiology experiment and 3D12A used in this experiment. The non-competitive immunoassay (Maggio, 1981; Kurstak, 1986) was used because of its high sensitivity in detecting small quantities of immunoglobulin. The limits of detection of this assay are 1 to 10 ng of protein.

Systemic Immune Response

This non-competitive assay was also used to determine that no systemic immune responses were mounted against the contents of the tubes. There was no evidence that antibodies to bovine collagen or murine IgG were present in the serum of the experimental animals, suggesting that the steady decline of antibody in the implants was not linked to immunologic clearance by reactive cells. N-CAM does not circulate in peripheral blood because it is a cell surface adhesion molecule specific for neurons and glia; therefore the presence of any systemic antibody would have been due to the presence of antibodies used in our implants. It is possible that the contents of the tubes were mechanically displaced by migrating Schwann cells or advancing nerve fibers.

Immunohistochemistry and Electron Microscopy

In cross-sections of the 10 day experimental nerves, abnormal nerve gaps predominated. These gaps were larger and more frequent than gaps in control nerves. In the present study, control nerve morphology did not differ from that described by others (Daniloff *et al.*, 1986b; Gibson *et al.*, 1989b; Ramon y

Cajal; 1928b). The appearance of gaps in nerve tissues severely injured by transection has been described as an expected component of the initial stages of regeneration (Ramon y Cajal, 1928b). This led us to conclude that the application of our antibody with anti-N-CAM like activity interfered with the normal abilities of injured nerves to regenerate by blocking cell binding mediated by N-CAM.

At 30 days post-surgery, morphology of control nerves reflected anticipated reorganization. This was depicted by the absence of gaps and the presence of axons in the distal stump. In experimental nerves, gaps, specifically between apposing Schwann cells, continued to be observed. No disruptions were observed between Schwann cells and axons. In the companion physiologic study, we found that the disruption of functional recovery was temporary and that by 30 days after surgery, somatic muscle control approached that of control nerves.

The present data offer a rationalization for the observed perturbation of function seen in early stages with muscle-evoked potentials. Furthermore, since antibodies disrupted only Schwann cell-Schwann cell interactions, the contributions of N-CAM to the process of regeneration may be through selective control of Schwann cell-Schwann cell adhesion. Morphological analyses of the thick sections taken at 60 days post-surgery suggest that myelin may be present in control nerves and to a lesser extent in experimental nerves. It remains to be

proven whether N-CAM is involved with the reformation of myelin, in addition to the growth of nerves and their responses to injury.

The availability of the N-CAM has been shown to be an essential component of the complex process of nerve regeneration. Since antibodies with anti-N-CAM like activity perturb regeneration, N-CAM potentially contributes to this process. The effect shown here can only be applied to the initial stages of regeneration and the contribution of N-CAM to later stages of regeneration remains to be shown.

CONCLUSIONS

Many studies have been done to determine substances available to aid in peripheral nerve regeneration. These include studies of the following compounds: laminin, fibronectin and collagen, growth factors, and cell adhesion molecules. All of these compounds are known to be involved at some stage of peripheral nervous system or central nervous system development; as such they have been implicated in regeneration.

Laminin is an integral basal lamina protein while fibronectin is a major component of basal lamina, connective tissue and extracellular matrix produced by fibroblasts. Growth factors support differentiation, maturation and survival of sympathetic and primary sensory neurons. NGF is involved in regulation of adhesion molecule expression (Thoenen and Barde, 1980).

External factors found to play a role in peripheral nervous system regeneration are the effect of a conditioning lesion or the application of a pulsed electromagnetic field to an animal. The conditioning lesion increases the rate of regeneration (Oblinger and Lasek, 1984; Sjöberg and Kanje, 1980); (1) by changes in non-neuronal cells, and trophic factor production due to Wallerian degeneration in the distal nerve and (2) the nerve cell body has already made necessary adjustments and axons have synthesized materials necessary for regeneration. The application of a PEMF was found to influence the synthesis of new polypeptides in rat sciatic nerve (Sisken *et al.*, 1989a).

In this study implants containing anti-N-CAM-like antibodies disrupted nerve regeneration between 10 and 30 days post-surgery. This affect appears to no longer be present in long-term (60 day) survivors. Muscle-evoked potentials appear to be a reliable way to estimate the extent of nerve regrowth. They are a non-invasive method of determining return of muscle function. We found that at 20 days, there was no return of muscle function as determined by comparing MEPs of control vs experimental nerves.

No significant changes were found in the levels of systemic rat antibody produced in reaction to the implanted murine IgG compared to pre-surgical levels. The presence of systemic rat antibodies to bovine collagen was not noted. These data suggest that no systemic immune responses were mounted against either the murine IgG or the collagen component of the implants.

No cell-mediated immunity was noted in the H & E stained distal nerve sections at the 10 day time periods indicating that macrophage clearance of antibody from the implant was not a major factor in the return of function seen at 30 days.

Immunohistochemistry at 10 days indicated that no neurofilaments were regrowing through the implant at this time. At 30 days, coinciding with the return of somatic muscle function, neurofilaments were beginning to be seen in the distal nerve. Electron micrographs confirm that gaps were present between Schwann cells and not between Schwann cells and axons. This implied that N-

CAM mediated Schwann cell-Schwann cell interactions in regenerating peripheral nerves and not Schwann cell-axon interactions. Since anti-N-CAM like antibodies perturbed regeneration temporarily, and Schwann cells are necessary for nerve regeneration, anti-N-CAM like antibodies disrupted this regeneration possibly by interacting directly with Schwann cell adhesiveness to each other. By blocking the N-CAM protein to perturb regeneration, we established its potential contribution to the general process.

The implantation of the antibody in this study around transected adult sciatic nerves delayed the reinnervation of target muscles for a period of between twenty and thirty days. No systemic immune responses to the implants were detected, however, electrophysiological recordings of the normal nerve indicate that there was a systemic effect at 20 days. It is possible that minute quantities of circulating antibodies disrupted Schwann cell-Schwann cell or Schwann cell-axon interactions in normal nerves. Since N-CAM is localized to nodes of Ranvier and the neuromuscular junction, and possibly related to maintenance of mature function, systemic antibody may have affected transmission at a distance as suggested by the latency data. This effect was no longer present at 30 days. These data implicate cellular interactions mediated by N-CAM, at least during the early stages of regeneration, as potential components of the regeneration process.

These results are strengthened by three observations from studies of surgical repair of nerve damage. Entublization of transected nerves with inert plastic has been shown to be an excellent method of intervention (Gibson and Daniloff, 1989a). It has also been shown that silastic tubes are more effective enhancers of early mammalian nerve regeneration than three other commonly used surgical methods (Gibson, *et al.*, 1989b). Furthermore, it has been shown that substrates such as laminin and collagen enhance rodent nerve regeneration (Gibson, *et al.*, 1989b). Therefore, the conditions of our entubulization procedure should have enhanced the potential for nerve regeneration in every animal, except for one factor: the antibody with anti-N-CAM like activity.

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APPENDIX A

Reagents and Gel Preparation for SDS-PAGE Slab Gels

Reference: Laemmli, 1970

A. **Acrylamide/Bis** (30% T, 2.67% C)(AMRESCO, Solon, OH)

146 g acrylamide (29.2 g/100 ml)
4 g N'N'-Bis-methylene-acrylamide (0.8 g/100 ml)

Make to 500 ml with distilled water. Filter and store at 4°C in the dark (30 days maximum).

Or substitute Bio-Rad Preweighed Acrylamide/Bis 37.5:1 mixture (Catalog No. 161-0112 30g)(Catalog No. 161-0106 200g).

B. **1.5M Tris-HCL, pH 8.8** - separating gel buffer

for 100 ml:

18.15 g Tris base (TRIZMA)
90 ml millipore distilled water

Adjust to pH 8.8 with concentrated HCL. Make to 100 ml with distilled water and store at 4°C.

C. **0.5M Tris-HCL, pH 6.8** - stacking gel buffer

for 100 ml:

6.0 g Tris base (TRIZMA)
80 ml millipore distilled water

Adjust to pH 6.8 with concentrated HCL. Make to 100 ml with distilled water and store at 4°C.

D. **Ammonium Persulfate**, 10%, (Bethesda Research Laboratories, Gaithersburg, MD)(Catalog No. 5523UA, Lot No. ACD209).

Make immediately prior to using:

0.1 g ammonium persulfate
0.9 ml distilled water

E. **Sodium Dodecyl Sulfate** - 10%

5 g SDS; make to 50 ml with distilled water.

F. **TEMED** (AMRESCO, Solon, OH; Catalog No. P0076156, Lot No. 188011)

use at room temperature, add just before ammonium persulfate

G. **10X Electrode Buffer** - for running electrophoresis in tank

Tris (TRIZMA, trisbase)	90 gm
Glycine	432 gm
SDS	30 gm

Make to 3 liters with distilled water. Dilute 100 ml to 1 liter for one run.

APPENDIX B

Western Blot on Nitrocellulose Paper

Reference: Towbin *et al.*, 1979

Reagents: **Biotinylated Goat anti-mouse**

Cappel #8711-3731 — Organon Teknika Corp.
1230 Wilson Drive
West Chester, PA 19380

Streptavidin-horseradish peroxidase

KPL #143000 — Kirkegaard & Perry, Inc.
2 Cessna Court
Gaithersburg, MD 20878

Solutions:

a. **EPBS (ELISA phosphate buffered saline)**

solution A	4 liters	solution B	1 liter
NaCl	8.0 g	KCL	0.2 g
KH_2PO_4	1.3 g	NaCl	32.0 g
KCL	0.8 g	Na_2HPO_4	5.4 g

Make up solution A and solution B in distilled water. Add solution B to solution A until pH reaches 7.4 (approximately 125 ml of solution B to 500 ml of solution A). Filter sterilize.

b. **Substrate**

1. Add 22.5 ml distilled water to 2.5 ml 0.5 M Tris-HCl (pH 6.8).
2. Add 15 mg 4-chloro-1-napthol in 5 ml methanol.
3. Add 10 ul 30% hydrogen peroxide.
4. Test substrate in streptavidin and add 2-4 ul peroxide if too weak.

c. **Alternate Substrate** TMB Membrane Peroxidase Substrate System

(3,3',5,5'-tetramethylbenzidine)
Kirkegaard & Perry, Inc., Gaithersburg, MD
catalog # 50-77-00

Procedure:

a. Block strips with 2 ml per lane of EPBS with 10% NGS for 1 hour on rocker platform.

b. Wash twice with EPBS (no additives) on rocker platform, 2 ml per lane.

c. Put 2 ml per lane of monoclonal supernatants on and incubate on rocker platform overnight. Do not dilute supernatants.

d. Wash 5 times with 2 ml per lane EPBS, 5 minutes for each wash.

e. Put on 1 ml per lane Cappel goat anti-mouse biotinylated 2nd antibody made in EPBS plus 10% goat serum (1:500 dilution) and incubate for 3 hours on rocker platform.

f. Wash 5 times with 2 ml per lane EPBS, 5 minutes for each wash.

g. Put on 1 ml per lane Streptavidin-Horseradishperoxidase made in EPBS plus 10% goat serum (1:750 dilution) and incubate for 1.5 to 2 hours on rocker platform.

h. Wash 5 times with 2 ml per lane EPBS, 5 minutes for each wash.

i. Add 1 ml per lane substrate and wait until color is of desired intensity.

j. Wash with distilled water and let dry.

APPENDIX C

Recipes for HiTrap™ Protein A Affinity Column

Source: Pharmacia LKB Biotechnology, Inc.
Piscataway, NJ 08855
Code No. 17-0402-01
Lot No. RC 13916

Reference: Kessler, S.W. 1975,1976; Richman *et al.*, 1982.

Buffer A: wash buffer
50 mM Tris, pH 8.6

Dissolve 3.03 gm Tris into 500 ml distilled H_2O. Adjust pH with 1N HCL to pH 8.6.

Buffer B: elution buffer
0.1 M citric acid, pH 3-6

Dissolve 2.94 gm citric acid into 100 ml distilled H_2O. Adjust pH with concentrated (6N) HCL to 3.1.

Buffer C: neutralization buffer
1.0 M Tris, pH 9.0

Dissolve 12.11 gm Tris into 100 ml distilled H_2O. Adjust pH with 1N HCL to 9.0.

* All buffers should be filter sterilized (0.45 μm) before application to the column.

APPENDIX D

Recipes for MAbTrap™ G Affinity Chromatography

Source: Pharmacia LKB Biotechnology, Inc.
Piscataway, NJ 08855
Code No. 17-0701-01
Lot No. TOA003

Reference: Björck and Kronvall, 1984

Buffer A: binding buffer
0.2 M sodium phosphate, pH 7.0

Dissolve 2.4 gm NaH_2PO_4 into 100 ml distilled H_2O. Adjust pH with 1N HCL to 7.0.

Buffer B: elution buffer
1.0 M glycine-HCL, pH 2.7

Dissolve 75 mg glycine into 100 ml distilled H_2O. Adjust pH with 1N HCL to 2.7.

Buffer C: neutralizing buffer
1.0 M Tris-HCL, pH 9

Dissolve 12.11 gm Tris into 100 ml distilled H_2O. Adjust pH with 1.0N HCL to 9.0.

* All buffers should be filter sterilized (0.45 μm) before application to the column.

APPENDIX E

Recipes for Avid AL Mini-Column

Source: BioProbe International, Inc.
The Nest Group
43 Valley Road
Southboro, MA 01772
800-347-6378
Catalog No. 5325-003, Lot No. 1-071-2

References: Khatter *et al.*, 1991; Füglistaller, 1989; Gassmann *et al.*, 1990.

Buffer A: wash buffer
0.01 M sodium phosphate, 0.15 M sodium chloride, 0.02% sodium azide, pH 7.4

Dissolve 0.23 gm NaH_2PO_4 (1.9 mM) and 1.15 gm Na_2HPO_4 (8.1 mM) and 9.0 gm NaCl (154 mM) into 1 liter of distilled H_2O. Adjust pH to 7.4 with 1N HCL.

Buffer B: elution buffer
0.05 M sodium acetate, pH 2.8

Dissolve 0.68 gm sodium acetate into 75 ml of distilled H_2O water. Add 25 ml glycerol. Adjust pH to 2.8 with concentrated (6N) HCL.

Buffer C: regeneration buffer
20 parts methanol, 80 parts 1% acetic acid

Add 5 mls of glatial acetic acid to 495 mls of distilled H_2O. Take 400 mls of this mixture and add 100 mls of methanol.

Buffer D: neutralizing buffer
1.0 M Tris Base, pH 9.0

Dissolve 12.11 gm of Tris into 100 mls of distilled H_2O. Adjust pH to 9.0.

* All buffers should be filter sterilized (0.45 μm) before putting on the column.

APPENDIX F

DEAE-Cellulose Anion Exchange Chromatography

Source: Whatman Specialty Products Division
P O Box 1359
Hillsboro, OR 97123-9981
Catalog No. 4057P050
Batch No. 1152012

Reference: Fahey and Terry, 1979; Miller *et al.*, 1991.

Buffer 1) 10X 0.175 M $NaPO_4$, pH 6.3

a) Dissolve 26.8 gm Na_2HPO_4 in 500 mls of distilled H_2O.

b) Dissolve 55.2 gm NaH_2PO_4 in 2000 mls of distilled H_2O.

c) Take 440 mls of A, add 1560 mls of B and add 280 mls of distilled H_2O.

d) Adjust pH to 6.3 with 1N HCL.

Buffer 2) 0.175 M $NaPO_4$ plus 0.05 M NaCl, pH 6.3

a) Make PBS as above buffer 1.

b) Add 2.92 gm NaCl/liter of buffer.

c) ADjust pH to 6.3 with 1N HCL.

* All buffers should be filtered (0.45 μm) before application to a column.

APPENDIX G

N-CAM Enzyme-Linked Immunosorbent Assay Procedure for Antibody Screening

	1	2	3	4	5	6	7	8	9	10	11	12
A	1H4 .119	1E6 .150	1A7 .224	1C10 -.005	1A11 -.048	1D11 -.048	1F12 -.087	2C1 -.097	2A2 0.100	2B2 -.019	2C3 .026	2G5 0.025
B	2F8 .597	2F10 .103	2E11 .033	2F11 0.021	2G11 .002	2F12 .025	3A1 -.002	3B1 .286	3G1 -.022	3B4 -.022	0% viability	3C4 -.072
C	3B5 -.062	3F5 .653	3A6 .069	3B6 .065	3A7 -.001	3B7 .015	3B8 -.023	3F8 -.049	3C10 -.035	3F10 -.021	3F11 -.072	4A2 .118
D	4F3 -.086	4C4 -.027	4D4 .030	4B6 .089	4E6 -.021	4B8 -.037	4G7 -.006	4E9 -.054	4F9 -.050	4G9 -.033	4G6 -.049	4B10 .215
E	4E10 .082	4H10 .021	4E12 -.048	15G8 >1.29	THY 1.1 .121	THY 1.1 .005	THY 1.1 -.014	THY 1.1 -.001	THY 1.1 -.032	THY 1.1 .001	THY 1.1 .015	THY 1.1 -.037
F	0% viability	0% viability	3D12A 1 .450	3B1A 2 .579	3D12A 1 >1.607	3D12A 1 >1.634	3B1A 2 >1.79	3B1A 2 >1.227	0% viability	0% viability	0% viability	0% viability
G	Blank .005	Blank -.009	Blank .058	Blank -.040	Blank .067	Blank -.056	Blank .019	Blank -.015	Blank -.051	Blank -.092	Blank -.052	Blank -.002
H	Blank .034	Blank -.036	Blank -.023	Blank .044	Blank .144	Blank -.027	Blank -.069	Blank -.040	Blank -.035	Blank -.040	Blank -.025	Blank .064

Blanks are 10% NGS in PBS. 15G8 = (+) control. Thy-1 = (-) control.
Each box contains clone identification and antigen/antibody reaction.

VITA

She received her Doctor of Veterinary Medicine from Louisiana State University in May 1990, her Master of Science degree from Louisiana State University in May 1985 and her Bachelor of Science degree from California Polytechnic State University in June 1979. Laura Remsen currently resides in Portland, Oregon.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Veterinary Medical Sciences

Title of Dissertation: Contributions of Monoclonal Antibodies with Anti-Neural Cell Adhesion Molecule Like Activity to Peripheral Nerve Regeneration.

Approved:

Joanne K Davis
Major Professor and Chairman

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November 23,1992